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(57) Abstract

The present invention relates to a mammalian expression system for the production of recombinant heteromeric proteins, preferably antibodies, and more preferably antibody fusion proteins, such as antibody-cytokine fusion proteins, and fragments thereof by means of oligocistronic expression vectors which are under the control of a strong promoter/enhancer unit, a selection marker gene and at least two IRES elements. The heteromeric fusion proteins can be produced in a robust and stable process in excellent yields.

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OLIGOCISTRONIC EXPRESSION SYSTEM FOR THE PRODUCTION OF HETEROMERIC PROTEINS

The present invention relates to a mammalian expression system for the production of recombinant heteromeric proteins, preferably antibodies and antibody fusion proteins such as antibody-cytokine fusion proteins and fragments thereof, by means of tri- or oligocistronic expression vectors which are under the control of a strong promoter/enhancer unit and which contain a selection marker as one of the cistrons. This selection marker guarantees together with at least two IRES elements a robust and stable production of the heteromeric proteins in excellent yields.

Background of the invention

For the expression of herteromeric proteins in mammalian cells such as antibody molecules traditionally two vectors have been used which frequently leads to unpredictable overexpression of one of the protein chains in comparison with the second one. Where one chain is relatively overexpressed the cells begin to suffer resulting in instability of production and/or in purification problems (e.g. light chain dimers). One traditional way to overcome this problem is to cotransfer the vectors in a well defined ratio into the host cells. This requires that the plasmid copies are accepted and integrated simultaneously and stable, and that the plasmid ratio remains constant during cell division. Only for a few systems satisfying results were obtained up to now.

Another traditional way is to use independent transcription units located on one plasmid. Thus, the different genes are present on the vector in a correct ratio. Provided that promoters of comparable strength are used equal amounts of the desired protein chains should be obtained. However, different stability and translation efficiencies of the mRNAs which are coding for the different proteins, and different transcription efficiencies of the genes lead to an unequal synthesis of the desired protein chains.

To avoid these problems di- and multicistronic vectors were developed recently. In such systems the gene units used (coding for the desired proteins, cistrons) are under the control of one single promoter. Normally, only the first cistron located at the 5' terminus is translated efficiently in eucaryotes since the initiation of the translation occurs according to the "cap"- dependent mechanism. The following cistrons are translated insufficiently or not at all. It has been found that the translation of the following cistrons in multicistronic systems can be initiated and pushed by using sequences having no "cap" structure. Such sequences are obtainable from non-translated sections of some viruses, such as poliovirus and encophalomyocarditis virus (Jang et al., 1988, J. Virol. 62:2636; Jang et al., 1989, J. Virol. 63: 1651; Pelletier und Sonnneberg, 1988, Nature 334:320). Within the virus sequences a short section which is not tranlated and called IRES (internal ribosomal entry site) can be used to allow translational reinitiation independent on the cap. Such sequences have to be interspersed between the cistrons to make a multicistronic mRNA functional. IRES sequences do not influence the "cap"-dependent translation of the first cistron. However, it was found that the "cap" dependent translation is, as a rule, more effective than the IRES-dependent translation which means that the proteins are expressed in a non-stoichiometric ratio and, finally, leads to a loss of stability. Thus, it is very difficult to produce two or more proteins in equimolar ratios even with means of a bi- or oligocistronic expression unit. Biscistronic expression systems and vectors, respectively, using non-antibody genes are known (e.g. Dirks et al., 1993, Gene 128:247). In most of these systems a gene coding for a selection marker was used as second cistron. International patent publication WO 94/05785 discloses a general teaching of expression units in which more than one IRES element can be theoretically inserted into the vector construction. In detail, however, only a bicistronic expression system is described using well defined genes, namely encoding PDGF chains A and B (platelet derived growth factor) separated by an IRES containing unit. No selection marker is used in this system.

It has not been reported until now that heteomeric proteins such as antibody heavy and light chains have been expressed in stoichiometric and stable formation by tri- or oligocistronic systems. It has not been reported, furthermore, that the use of a selction marker as one of the cistrons leads to transformed cells which have an extraordinaryly high stability.

Equimolar and stable production of the heteromeric protein chains, such as the heavy and light chain of antibodies, is a prerequisite for a correct association and folding of the two chains, and, therefore, for a correct steric conformation which is important in order to achieve an optimal biological activity of the associated heteromeric protein or peptide chains.

In the case of an antibody fusion protein, the biologically active ligand for an antibody-directed targeting should induce the destruction of the target cell either directly or through creating an environment lethal to the target cell. The biologically active ligand can be a cytokine such as IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-13, IFNs, TNFα or CSFs. These cytokines have been shown to elicit anti-tumor effects either directly or by activating host defense mechanisms (e.g. Mire-Sluis, TIBITECH, 11:74). For instance, IL-2 is considered the central mediator of the immune response. IL-2 has been shown to stimulate the proliferation of T- cells and NK-cells and to induce lymphokine-activated killer cells (LAK). IL-2 enhances the cytotoxicity of T-cells and monocytes. TNF alpha has found a wide application in tumor therapy, mainly due to its direct cytotoxicity for certain tumor cells and the induction hemorrhagic regression of tumors. In addition TNF alpha potentiates the immune response: it is a costimulant of T-cell proliferation, it induces expression of MHC class I and II antigens and TNF alpha, IFN and IL-1 secretion by macrophages. However, most of the known cytokines activate effector cells, but show no or only weak chemotactic activity.

Chemokines, however, are chemotactic for many effector cells and enhance their presence at the tumor site and induce a variety of effector cell functions (e.g. Miller and Krangel, 1992, "Biology and Biochemistry of the Chemokines,...", Critical Reviews in Immunology 12:17). Examples for suitable chemokines according to the invention are IL-8 and MIP 2α and MIP 2ß which are members of the C-X-C chemokine superfamily (also known as small cytokine superfamily or intecrines).

Epidermal growth factor (EGF) is a polypeptide hormone which is mitogenic for epidermal epithelial cells. When EGF interacts with sensitive cells, it binds to membrane receptors (EGFR). The EGFR is a trans-membrane glycoprotein of about 170 kD, and is a gene product of the c-erb-B proto-oncogene.

The murine monoclonal antibody mAb425 was raised against the human A431 carcinoma cell line (ATCC CRL 1555; US 5,470,571) and was found to bind to a polypeptide epitope on the external domaine of the EGFR. It was found to inhibit the binding of EGF and to mediate tumor cytotoxicity in vitro and to suppress tumor cell growth of epidermal and colorectal carcinoma-derived cell lines in vitro (Rodeck at al., 1987, Cancer Res., 47:3692).

Humanized and chimeric version of mAb425 are known from WO 92/15683. Fusion proteins of mAb425 (as a whole or fragments thereof) and cytokines or chemokines are described in European patent publications EP 0659 439 and EP 0706 799.

Summary of the invention

Thus, it is an object of the present invention to provide an expression system suitable for the stable production of a heteromeric protein, preferably an antibody, and more preferably an antibody fusion protein, which avoids the problems of the prior art systems as described above.

It has been found as a result of this invention that a proper expression of these heteromeric proteins can be achieved by using oligocistronic expression units comprising at least two IRES elements where the different heteromeric chains, e.g the heavy and light protein chain of an antibody, are cotranslated from one mRNA molecule comprising a sequence encoding a selection marker. The strength of the effect caused by the selection marker in this system is surprising and could not be expected compared with usual expression systems of the prior art. The effect is especially strong when the gene encoding the selection marker is located at the end of all cistrons each separated by IRES units. This is not the case if the selection pressure is removed or if the selection marker is used in traditional expression vectors. Using the selection marker as last cistron forces the cell to produce the linked protein / proteins.

The constructs according to the invention allow equimolar production of the heteromeric protein chains and guarantee selection and stable, long-term expression of the optimal production clones by concomittant expression of the selection marker, because only those clone will grow under selection pressure which express the entire cistronic expression unit.

It has been found that the combination of a selection marker gene and an IRES sequence located behind a bicistronic unit (to form a tricistronic unit) comprising the sequence coding for the light chain of an antibody, an IRES sequence and a sequence coding for a fusion protein consisting of the heavy

chain of an antibody fused to another biologically active protein, such as a cytokine or chemokine, is very advantageous with respect to a stable expression in excellent yields.

It is an objective of the present invention to provide a new expression system for eucaryotic cells which ensures a stable, reproducible and robust production process for recombinant single and multi-chain protein complexes such as antibodies or, especially, antibody-cytokine fusion proteins.

The present invention relates to a mammalian expression system for the production of heteromeric proteins, preferably recombinant antibodies and more preferably antibody fusion proteins such as antibody-cytokine fusion proteins and fragments thereof.

The invention relates, preferably, to such a expression system which is able to produce antibody fusion proteins or fragments thereof, wherein the antibody binding sites are directed to the human EGF-receptor and the antibody is covalently linked to a biologically active ligand such as a growth and/or differentiation factor, above all TNF alpha, or IL-2. The invention discloses a set of vectors which comprise oligocistronic, preferably tri- and tetracistronic expression units driven by a single strong promoter hybrid linked to genes encoding protein chains of the light chain, the heavy chain and the active ligand and, additionally a selection marker in the promoter-distal position. Cotranslation of these proteins from one oligocistronic mRNA guarantees strict coupling of expression and allows stoichiometric production of protein chains.

Therefore, it is an object of the invention to provide an oligocistronic expression vector suitable for the production of a heteromeric protein consisting of at least two protein chains in a mammalian host cell comprising

(i) a promoter / enhancer sequence,

(ii) a sequence encoding a first chain of the heteromeric protein or a fragment thereof,

(iii) a sequence encoding a second chain of the heteromeric protein or a fragment thereof,

(iv) optionally a sequence encoding a third or further chain of the heteromeric protein or a fragment thereof,

(v) a sequence encoding a selection marker, and

(vi) at least two sequences comprising a 5'-UTR poliovirus sequence containing an IRES element.

It has been found now that the order of the genes located in the vector construct is important with respect to the described advantageous effects. Thus, especially, the gene coding for the selection marker should be located as last cistron within the vector construct. Additionally, in the case of an antibody, the gene encoding the light chain of the antibody should be located in upstream position before the gene coding for the heavy chain.

Therefore, it is a preferred object of the invention to provide said expression vector, wherein the sequences (i) to (vi) are in the following order from upstream to downstream progression of said vector construct:

(1) a sequence comprising the promoter / enhancer sequence (i),

(2) a sequence comprising the sequence encoding a first chain of the heteromeric protein or a fragment thereof (ii),

(3) a sequence (vi) comprising a first IRES element,

(4) a sequence comprising the sequence encoding a second chain of the heteromeric protein or a fragment thereof (iii),

(5) a sequence (vi) comprising a second IRES element,

(6) optionally a sequence comprising the sequence encoding a third or further chain of the heteromeric protein or a fragment thereof (iv), a sequence comprising a third or further IRES element (vi) included,

(7) a sequence comprising the selection marker (v).

The advantage of this system is also shown in Fig. 17 and 18. Under selection pressure the clones produce in a stable manner the different chains of the heteromeric protein but without selection pressure or "wrong" position of the selection marker the stable productivity is rapidly lost. The greatest advantage of the system is that (heteromeric) proteins can be expressed which can be toxic to the host cells like proteases, glutamate receptor subtypes and serotonin receptor subtypes or antibody fusion proteins wherein the non-antibody partner is normally highly toxic for the host cells.

Preferably, a corresponding expression system is object of the invention, wherein the sequence (ii) encodes the light chain and the sequence (iii) comprises a sequence encoding the heavy chain of a monclonal antibody or a fragment thereof. However, the teaching of this invention is also applicable for heteromeric proteins other than antibodies, for heteromeric proteins having more than two chains, and even normal (one-chain) proteins having toxic activity against the host cell and, finally, heteromeric proteins (e.g. antibody fusion proteins) having strong toxic activity caused by a part of said heteromeric protein..

Furthermore, a corresponding expression system is object of the invention, wherein the sequence (iii) consists of two sequences (iiia, iiib), wherein (iiia) encodes the heavy chain of an antibody or a fragment thereof and (iiib) encodes a biologically active ligand, such as a cytokine or a chemokine or a fragment thereof, in order to form a fusion protein.

It has been found, additionally, that such expression vector constructs are preferred, and therefore, object of the invention, wherein the sequence of (iiia) is shortened at its C- terminus and the sequence (iiib) at its N-terminus each by 1 to 15 amino acids.

A special and preferred embodiment of the invention is a tricistronic expression vector as defined above and in the claims, wherein the sequence (iiia) and the sequence (iiib) are linked directly in order to encode a fusion protein.

In addition the expression vector according to the invention may, optionally, contain eucaryotic sequence elements such as SAR/MAR elements to further increase production and stability of the system. The expression of certain genes has been reported to respond positively to butyrate. The stimulatory effect of butyrate is largest if one or two scaffold/matrix-attached regions (SAR/MAR elements) are present adjacent to the gene (Schlacke et al., 1994, Biochemistry 33:4197). Only after integration of the constructs in to the genome of the host cell these regions increase the expression of adjacent genes in an orientation- and position-independent fashion. Gene activation causes the apparent loss of nucleosome structure ahead of the SAR element and a similar change has been demonstrated by the action of butyrate. Presence of both SARs and butyrate act synergistically in enhancing gene expression (Klehr et al. 1992, Biochemistry 31:3223).

Therefore, an expression vector defined above and in the claims is object of the invention, comprising, additionally, one or two, preferably two, SAR elements. Preferably, one SAR element is located in front of the promoter/enhancer region the second one behind the sequence encoding the selection marker. However, other locations are also possible.

Preferably, the invention relates to antibody fusion proteins, wherein the non-antibody protein is a biologically active protein. Preferably, such expression

vectors are object of the invention, wherein a sequence (iiib) is used which encodes a cytokine or chemokine such as TNF alpha, IL-2 and IL-8.

Above all, such expression vectors are object of the invention, wherein the sequences (ii) and (iii) comprise sequences coding for the light and heavy chain of a monoclonal anti-EGFR antibody, preferably, humanized monoclonal antibody 425 (mAb425) or fragments thereof. However, the invention is not restricted to anti-EGFR antibody or mAb425, respectively, but includes also any other monoclonal antibodies directed to a variety of specificities, for example mAb361.

As an especially preferred embodiment it is object of the invention to provide an expression vector comprising the following units in the given order: the CMV/MPSV promoter/enhancer sequence followed by the sequence encoding the mAb425 light chain, followed by the sequence from 5'-UTR poliovirus containing an IRES element, followed by a fusion gene encoding a fusion protein consisting of the heavy chain of humanized mAb425 and fused at its C-terminus the sequence encoding TNF alpha or IL-2, followed by another IRES element from 5'- UTR poliovirus, followed by a sequence coding for puromycin acetyl transferase as selection marker and, finally a nucleotide sequence derived from the polyadenylation signal of SV40.

Furthermore, the well-defined expression vector comprising the nucleotide and amino acid sequences depicted in Figure 15 is object of this invention.

Additionally, it is an object of the invention to provide an expression system comprising a mammalian host cell transformed with an expression vector specified above and in the claims, preferably, wherein the host cell is CHO or BHK.

Finally, it is an object of this invention to make available a process for the production of a heteromeric protein, preferably an antibody, especially an antibody

fusion protein, especially a mAb425/TNF alpha or mAb425/IL-2 antibody fusion protein, or fragments thereof, by cultivating the host cells of an expression system as specified above and in the claims in a suitable nutrient and separating, if a tricistronic vector is used, the complete and active antibody fusion protein from the cells and / or the medium.

Brief Descriptions of the Figures

Fig. 1 (a-e):

Expression plasmids for the generation of tricistronic expression vectors. AmpR= Ampicillin resistance gene; IRES = Poliovirus derived internal ribosomal entry site; MPSV = Promoter/Enhancer; CMV = Cytomegalo virus promoter; Puromycin R = Puromycin resistance gene; SV 40 pA = SV 40 polyadenylation site.

Fig. 2:

Stability of BHK-21 mAb425CH1 clones.Stability of three different clones was determined over the time period indicated. The production of mAb425CH1 fusion protein of 10^6 cells/ml per 24 hrs was determined in an anti-Ig based ELISA. Cells were cultured in medium with (+P) or without (-P) Puromycin.

Fig. 3:

Stability of a BHK21 mAb425CH1-TNFα clone. Cells were cultured in DMEM medium for 89 days without selection pressure. The production of mAb425CH1 fusion protein of 10^6 cells/ml per 24 hrs was determined in an anti-Ig based ELISA.

Fig. 4:

Stability of a BHK21 mAb425CH3-IL-2 clone. Cells were cultured in DMEM medium for 48 days without selection pressure. The production of mAb425CH1 fusion protein of 10^6 cells/ml per 24 hrs was determined in an anti-Ig based ELISA.

Fig. 5:

SDS PAGE of purified mAb425CH3-IL-2. Lane 1: mAb425CH3-IL-2; Lane 2: IgG1 control antibody. Proteins were run on a 4 to 15% gradient gel (Phast System, Pharmacia) and stained with Coomassie.

Fig. 6:

FACS analysis of purified mAb425CH3-IL-2. The human EGF-R-positive carcinoma cell line A431 was incubated with the indicated antibody concentrations. Two different preparations of purified mAb425CH3-IL-2 were compared with purified mAb425 reference antibody.

Fig. 7:

Determination of IL-2 activity of purified mAb425CH3-IL-2. IL-2-dependent mouse CTLL2 cells were incubated with mAb425CH3-IL-2 or rec. human IL-2 (WHO Standard). Concentrations of fusion protein are shown as mAb425 equivalents, determined by an ELISA based on an anti-idiotypic antibody specific for mAb425. $5x10^4$ were cultured for 2 days and pulsed with 0,5 µCi ^{3}H-Thymidine 18 hrs before harvesting.

Fig. 8:

pMCLDHAP tricistronic vector for the expression of mAb425CH3-TNFα. AmpR= Ampicillin resistance gene; IRES = Poliovirus derived internal ribosomal entry site;.MPSV = Promoter/Enhancer; CMV = Cytomegalo

virus promoter; Puromycin R = Puromycin resistance gene; SV 40 pA = SV 40 polyadenylation site.

Fig. 9:

Stability of a BHK21 mAb425CH3-TNFα clone. Cells were cultured in DMEM medium for 48 days without selection pressure. The production of mAb425CH1 fusion protein of 10^6 cells/ml per 24 hrs was determined in an anti-Ig based ELISA.

Fig. 10:

Integrity of expression vector DNA in the absence of selective pressure. BHK-21 cell clones transfected with pMCLDHAP and expressing mAb425CH3-TNFα fusion protein were either cultivated under puromycin pressure (+) or grown in the absence of puromycin (-) for the indicated times. Graph A shows antibody fusion protein secretion (μg IgG/ml x 24 hr). B is a Southern blot of chromosomal DNA prepared from cells which were taken at the indicated times. The DNA was restricted with PstI and hybridized with a labelled PstI fragment from pMCLDHAP (1231 bp) encompassing part of the heavy chain fusion protein encoding cDNA (hc). mbh1 represents a single copy DNA fragment (1900 bp) of a hamster c-myc gene which was cohybridized using a specific probe (see example 7). Since both probes are labelled with the same specific activity and their length is similar, the intensity of the hc band corresponds to the copy number of the integrated expression plasmid.

Fig. 11:

FACS analysis of purified mAb425CH3-TNFα. The human EGF-R-positive carcinoma cell line A431 was incubated with the indicated antibody concentrations. Purified mAb425CH3-TNFα was compared with purified humanized mAb425 reference antibody.

Fig. 12:

Determination of TNFα activity of purified mAb425CH3-TNFα on MCF7 cells. The TNFα-sensitive and EGF-R negative human breast adenocarcinoma cell line MCF7 was used to determine the TNFα activity of the mAb425CH3-TNFa fusion protein. Concentrations of fusion protein are shown as mAb425 equivalents, determined by an ELISA based on an anti-idiotypic antibody specific for mAb425. humanized mAb425 and rTNFα are mixed at a ratio of 6:1 reflecting the molecular ration of both parts in the fusion protein. $5x10^4$ were cultured for 4 days and pulsed with 0,5 μCi ^3H-Thymidine 18 hrs before harvesting.

Fig. 13:

TNFα mediated cytotoxicity of purified mAb425CH3-TNFα is dependent on TNFα sensitivity. The TNFα-resistant and EGF-R-positive human carcinoma cell line A431 was used to determine the specificity of the mAb425CH3-TNFα fusion protein. Concentrations of fusion protein are shown as mAb425 equivalents, determined by an ELISA based on an anti-idiotypic antibody specific for mAb425. Humanized mAb425 and rTNFα are mixed at a ratio of 6:1 reflecting the molecular ratio of both parts in the fusion protein. $5x10^4$ were cultured for 4 days and pulsed with 0,5 μCi ^3H-Thymidine 18 hrs before harvesting.

Fig. 14:

mAb425CH3-TNFα is highly cytotoxic for EGF-R-positive and TNFα-sensitive human tumor cell lines. The human mamma carcinoma cell lines BT20 and the human melanoma cell line C8161 are both TNFα-sensitive and EGF-R-positive. Concentrations of fusion protein are shown as mAb425 equivalents, determined by an ELISA based on an anti-idiotypic antibody specific for mAb425. mAb425 and r TNFα are mixed at a ratio of 6:1 reflecting the molecular ratio of both parts in the fusion protein. $5x10^4$ were

cultured for 4 days and pulsed with 0,5 µCi ^{3}H-Thymidine 18 hrs before harvesting.

Fig. 15:

Complete nucleotid and amino acid sequence (coding regions) of mAb425CH3-TNFα as shown in Fig. 8.

Fig. 16:

Hystory of relevant vectors of the invention.

Fig. 17:

Stability of different antibody fusion protein cell clones (rBHK21mAb425-CH1-IL2). A = mAb425; stability of 3 different clones is tested. The production of fusion protein of 10^{6} cells / ml in 24 h is determined in the ELISA detecting the antibody part. Cells are cultured for the indicated days in medium with (+P) or without (-P) Puromycin.

Fig. 18:

Stability of the cell clone rBHK21mAb425-CH3-IL2698-8 with (CHO-M + P) and without (CHO-M - P) selection pressure (puromycin). The stability is tested for 70 days in culture. The production of protein of 106 cells / ml in 24 h is determined in an ELISA detecting the antibody part of the protein.

Detailed Description

Above and below the term "heteromeric protein" means a protein which natuarally consists of two or more chains. Only if the corresponding chains are associated and folded correctly the full biological activity of the heteromeric protein can bc obtained.

Above and below the term "mAb425CH1-" means an antibody construction containing the light chain, the variable region of the heavy chain, and the CH1 domain of the constant region of mAb425.

Above and below the term "mAb425CH2-" means an antibody construction containing the light chain, the variable region of the heavy chain, and the CH1 and CH2 domain of the constant region of mAb425.

Above and below the term "mAb425CH3-" means an antibody construction containing the light chain, the variable region of the heavy chain, and the CH1, CH2 and CH3 domain of the constant region of mAb425. This construct corresponds to the complete antibody.

Above and below, the term "a sequence encoding ....." does not mean exclusively the specific coding sequence, but may include also a sequence comprising said specific coding sequence, provided that no other statement is made.

Said additional sequences indicated above and coding for proteins [ii, iii (iiia, iib), iv, vi] can be prolonged or shortend each by 1 to 20 amino acids provided that the specific biological properties are not substantially amended. Prolongation can be caused, for example, by linker or leader peptides. Furthermore, the expression vector constructs according to the invention may contain introns which are not tranlated into amino acids. Prolongations and deletions of coding regions may occur, preferably, at the C- and / or N-terminus of the corresponding specific

peptide or protein. Preferred deletions according to the invention may occur at the C-terminus of the heavy chain of the antibody and the N-terminus of the biological ligand.

Furthermore, the invention includes also mutations and varients of the sequences indicated in detail having the same or a very similar biological activity. Such mutations and varients can be produced by accident (e.g. spontaneous mutations, natural radiation) or by intended chemical or physical activities.

The term "antibody fragment" means according to the invention an antibody fragment as defined above (mAb-CH1, mAb-CH2) as well as complete antibody (mAb-CH3) which is shortend by 1 to 20 amino acids at the C-terminus of its constant region.

The term "biological active ligand" means according to the invention any protein or peptide ligand which is effective against a target cell, above all, against a target cell which is recognized by the antibody part of the antibody fusion protein. The effect of the biological ligand may be, for instance, a toxic and/ or lysing and / or inhibiting one against the target cell, preferably a tumor cell. Examples of suitable biological active ligands are given above.

The term "biological activ ligand fragment" means according to the present invention a biological ligand (cytokines, chemokines) which is usually shortened by 1 to 20 amino acids at its N-terminus which is connected directly, or optionally via a linker peptide, to the (optionally shortened) C-terminus of the constant region of the antibody heavy chain.

All microorganisms, cell lines, plasmids, promoters, resistance markers, replication origins, restriction sites or other fragments or parts of vectors

which are mentioned in the description not directly in connection with the claimed invention are commercially or otherwise generally available. Provided that no other hints are given, they are used only as examples and are not essential with respect to the invention, and can be replaced by other suitable tools and biological materials, respectively.

The techniques which are essential according to the invention are described in detail below and above. Other techniques which are not described in detail correspond to known standard methods which are well known to a person skilled in the art, or are described mor in detail in the cited references and patent applications and in the standard literature (e.g. Sombrook et al., 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor; Harlow, Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor).

The selection marker according to the invention can be in principal any known selection marker suitable for high expression systems. Examples are enzymes such as puromycin-acetyl transferase or neomycin phosphotransferase. Puromycin-acetyl transferase is preferred according to this invention.

Alternatively, dominant acting genetic markers useful for monitoring gene transfer in mammalian cells that are based on procaryotic genes encoding key steps in the synthesis of the essential amino acids, such as tryptophane or histidine can be used. Under appropriate conditions, expression of these genes obviates the nutritional requirements for their respective amino acid products. Expression of the ß subunit of tryptophan synthase (trpB, EC 4.2.1.20) of Escherichia coli allows mammalian cell survival and multiplication in medium containing indole in place of tryptophane. The hisD gene of Salmonella typhimurium encodes histidinol dehydrogenase (EC

1.1.1.23), which catalyses the two-step NAD+-dependent oxidation of L-histidinol to L-histidine. In medium lacking histidine and containing histidinol only mammalian cells expressing the hisD gene survive. Use of these markers is advantageous over the use of antibiotics because for either trp or his selection the substitute nutrients indole or histidinol are readily available, inexpensive, stable, permeable to cells and convertible to the end product in a step controlled by one gene (Bode et al. 1995, Int. Rev. Cytol., R. Berezney & K.W. Jeon eds. Academic Press, Vol 162A:389)

As IRES sequences all sequences deriving from viral, synthetic origin or from cells can be used which allow an internal binding of ribosomes. Examples for such sequences are the 5'-UTRs elements from poliovirus type 1, 2 or 3 (picorna virus), from "encephalomyocarditis virus" (EMCV) (Sugimoto et al., 1994, BioTechnol. 12:694), from "Theilers murine encephalomyelitis virus" (TMEV), from "foot and mouth disease virus" (FMDV), from "bovine enterovirus" (BEV), and from "coxsackie B virus" (CBV).

The tri- or oligocistronic expression vector according to the invention works with a single strong promoter/enhancer unit. Examples for suitable promoters/enhancers are: CMV (Boshart et al., 1985, Cell 41:521); MPSV-LTR (Laker et al.,1987, Proc. Natl. Acad. Sci. USA 74,:8458); MPSV-CMV; RSV (Gorman et al., 1982, Proc. Natl. Acad. Sci. USA 79:6777); SV40 (Artelt et al., 1988, Gene 128: 247). The system MPSV(enhancer)-CMV(promoter of the cytomegalie virus) is the preferred unit according to the invention.

The fusion protein described in the examples contains a monoclonal antibody with specificity for the human EGF-receptor(EGFR). The monoclonal mAb425 was raised against the human A431 carcinoma cell line

and found to bind to a polypeptide epitope on the external domain of the EGFR. The heavy chain mAb425 antibody was fused C-terminally to cytokines/chemokines such as IL-2, IL-4, IL-7, TNFα and IL-8 as biologically active ligands. The constructs encoding these immuno-conjugates were generated with recombinant DNA technologies. As pointed out above, the immuno-conjugates contain the variable region of the antibody heavy chain and the CH1 domain of the constant region (antibody-CH1 conjugates), or the CH1 and CH2 domain of the constant region (antibody-CH2 conjugates) or the CH1, CH2 and CH3 domain of the constant region (antibody-CH3 conjugates) fused to the biologically active ligand. By addition of the appropriate light chain immunoconjugates can be generated which target antigen-bearing cells and deliver an active ligand to to a specific site in the body. The C-terminal amino acid sequence of the junctional region of CH1 and CH3 fusion proteins is not involved in any secondary structure elements according to the hypothetical computer model. In these regions several putative sites for proteolytic cleavage are present. In order to retain/increase chemical and biological stability these sequences can be shortened up to a limit where the biological activity of the ligand is lost. N-terminal cytokine sequences are frequently involved in receptor binding and biological activity, e.g. in human TNFα amino acid sequences between positions 11 and 35 appear to be critical for receptor binding and triggering of biological responses (Goh & Porter, Prot. Eng. 4:385, 1991). In those cases where loss of activity is caused by inaccessibility of relevant amino acids due to interference of the antibody part linker sequences can be introduced which consist of repetitive units containing amino acids which do not interfere with chemical stability and biological activity, e.g. see Curtis et al. Proc. Natl. Acad. Sci. USA, 88:5809, 1991.

In a preferred embodiment according to the invention a system of expression vectors is provided, which allows easy generation of expression vectors for synthesis of three proteins from a tricistronic expression unit.

In a preferred embodiment according to the invention tricistronic vectors have been constructed in which IgG light chain, heavy chain-cytokine fusion protein and a selectable marker are translated from one mRNA. Sequences of translation reinitiation elements (internal ribosomal entry sites = IRES) derived from the 5′-UTR′s of poliovirus, which mediate a cap-independed internal initiation of translation, are interspersed between the cistrons.

In a preferred embodiment according to the invention the tricistronic mRNA is transcribed from any strong promoter such as a single hybrid MPSV/CMV promoter/enhancer.

In a further preferred embodiment the selection marker may be puromycin acetyl transferase, neomycin phosphotransferase or procaryotic genes such as the ß-subunit of tryptophane synthase (trpB) derived from *E. coli* or the histidinol dehydrogenase (hisD) of *Salmonella typhimurium* or any resistance marker known in the art. The selection marker is preferably located in the promoter-distal position to ensure stable expression of the entire cistron.

In another preferred embodiment of the invention expression is further enhanced by inclusion of one or two, preferably two, scaffold/matrix-attached regions (SAR/MAR elements) into the expression vector. Expression can be synergistically by SAR/MAR elements and butyrat added to the medium.

In another preferred embodiment of the invention the protein sequence between both parts of the fusion protein can be shortened up to a limit where the biologically active ligand looses its activity.

In another preferred embodiment of the invention both parts of the fusion protein can be combined by introducing linker sequences which consist of repetitive units containing preferentially the amino acids alanin, glycin and serin.

Furthermore, it is an objective of the invention to manufacture said proteins such as immunoconjugates by transfering the expression vector which contains the tricistronic construct into appropriate host cells such as BHK-21 cells, CHO cells, SP2/0 cells or myeloma cells.

Generation of fusion protein constructs consisting of mAb425 and cytokines or chemokines has been disclosed in EP 0659 439 and EP 0706 799, respectively. Fusion proteins have been constructed on the basis of chimeric and humanized mAb425 with cDNAs encoding cytokines such as IL-2, IL-4, IL-7 and TNFα or chemokines such as IL-8 and MIP-2α and Mip2-ß fused to the CH1, or CH2 or CH3 domain of the constant region of the mAb425 heavy chain, respectively. The techniques used can be taken, for example from the two European patent publications indicated above which are incorporated in this application by reference.

The vector system according to the invention leads to an new and innovative production system for high expression of heterodimeric proteins in eucaryotic cells such as antibody-cytokine/chemokine fusion proteins. Light chain and heavy-chain cytokine/chemokine fusion are transcribed together with a selectable marker from one tricistronic mRNA. The advantage of this system is twofold: First, unpredictable overexpression of one of both chains

which frequently leads to instability of production and purification problems will be avoided because both chains will be produced at equimolar amounts. Secondly, coupling of product and selection marker in the promoter-distal position guarantees stable and longterm expression of the product. Taken together, the system described herein represents a robust process for production of complex proteins in eucaryotic cells employing different fermentation techniques.

Introduction of vector constructs for the expression of a monovalent immunoconjugate including only the CH1 domain or divalent immunoconjugates including the CH1 and CH2 and CH3 domains into host cells can be achieved by electroporation, DEAE dextrane, calcium phosphate, Lipofectin, protoplast fusion or any known method in the art. Any host cell type may be used provided that the recombinant DNA sequences encoding the immunoconjugate and the appropriate light chain are properly transcribed into mRNA in that cell type. Host cells may be mouse myeloma cells which do not produce immunoglobulin such as Sp2/0-AG14 (ATCC CRL 1581), NSO (Gaffe & Milstein, 1991, Meth. Enzymol. 73(B):3), P3X63Ag8.653 (ATCC CRL 1580) or hamster cells such as CHO-K1 (ATCC CCL 61), or CHO/dhFr- (ATCC CRL 9096), or BHK-21 (ATCC CCL 10). Selection for transfected host cells is done in the presence of the selection marker encoded by the third cistron of the tricistronic expression vector. Clones are analyzed for expression of immunoconjugates by EGF-receptor or cytokine-specific ELISAs. Selected clones are then further purified by limiting dilution cloning.

Examples

Example 1

Generation of basic vectors

The vectors pSBC-1 and pSBC-2 (Dirks et al., 1993, Gene 128:247) have been developed as monocistronic expression vectors. Both vectors contain the SV40 origin of replication, the SV40 early promoter, the SV40 19s splice donor and 19s acceptor, the SV40 polyadenylation signal, procaryotic sequences such as the origin of replication from ColE1 and the Ampicillin resistance gene. In addition pSBC-1 contains the internal ribosomal entry site sequence (IRES) of polio virus for the generation of dicistronic messenger RNAs when appropriately combined with pSBC-2. pSBC vectors were altered by replacing the promoter fragment (ClaI/XhoI) by a hybrid promoter/enhancer composed of an MPSV enhancer of 300 bp (ClaI/XbaI) (Dirks et al., Gene 128:247, 1993) and a PCR amplified huCMVpromoter fragment with XbaI and XhoI ends (bp 220-807 from HEIEE EMBL database) and by replacing the EcoRI-HindII polylinker by a HindIII-EcoRI polylinker to give pMC-1 **(Fig. 1A)** and pMC-2 **(Fig. 1B)**, respectively. Based on these vectors a set of vectors have been generated which allow generation of tricistronic expression vectors in a straightforward cloning strategy. The vectors pMC-1 and pMCC-1 **(Fig. 1C)** are identical except for the multi-cloning sites to facilitate insertion of restriction fragments. In these vectors the promoter-proximal cistron has to be inserted. pMC-2 and pMCC-2 **(Fig. 1D)** are also identical except for the multi-cloning site and allow expression of one protein chain, but do not contain a selection marker.

The vector pMC-2P **(Fig. 1E)** was created in several steps. First, the blunt-ended fragment of the puromycin resistance gene from pSV2pac (Vara et al. 1986, Nucl. Acid Res. 14:4617) was cloned into the NotI site of pMCC-1. In the resulting plasmid the XbaI/EcoRI was replaced by the analogous fragment from pMCC-2, thereby inserting a new NotI site. The resulting

plasmid is called pMCC-2P **(Fig. 1F)**. pMC-2P was created by exchanging the polylinker into an HindIII/EcoRI polylinker. pMC-2PS **(Fig. 1G)** was created by insertion of a scaffold-attached region sequence (SAR) of 800 bp from the human Interferon-ß gene as described (Mielke et al. 1990, Biochemistry 29:7475). All three vectors contain an IRES sequence followed by the selection marker, in this case Puromycin resistance.

After cloning of the respective DNA fragments encoding the protein chains to be expressed into the appropriate vectors generation of a tricistrion expression vector is performed as follows: A ClaI/NotI restriction fragment containing the promoter-proximal cistron followed by an IRES sequence is derived from the vectors pMC-1 or pMCC-1, respectively. A NotI/ClaI restriction fragment containing the second cistron followed by an IRES sequence and the selection marker is derived from the vectors pMCC-2P, pMCC-2, pMC-2P, and pMC-2PS. By combination of these two fragments a complete expression vector is generated.

Example 2

Cells and gene transfer

BHK-21 cells (A subclone of ATCC number CCL-10) were cultivated in DMEM supplemented with 10 % fetal calf serum (FCS), 20mM glutamine, 60 µg/ml penicillin and 100 µg/ml streptomycin.

Calcium phosphate transfections were carried out essentially as described before (Mielke et al. 1990, Biochemistry:29:7474). Minimally 5 µg of uncut plasmids were used without the addition of carrier DNA. Stable transfectants were selected and - where indicated - cultivated in medium containing puromycin (Sigma) at a final concentration where only cells expressing the Puromycin resistance marker can grow, e.g. 5 µg/ml for BHK-21 cells.

Example 3

Quantification of secreted antibody

10^6 cells/ml were seeded on 25 cm^2 culture flasks in serum free medium and incubated for 24 hours. Medium samples of these cultures were taken for quantification of secreted IgG-chains in a specific ELISA. For this purpose, 96 well immunoplates (Nunc) were coated with an affinity purified goat-anti-human IgG antibody (Fab' specific, Sigma# 1-5260). After incubation with serial dilutions of medium samples, the bound antibody contained in these samples was detected by application of a peroxidase-conjugated affinity pure goat-anti-human IgG antibody (Dianova#109-035-088) and subsequent staining with ortho-Phenyldiamine-dihydrochloride (OPD)/H_2O_2. Quantification was made possible by simultaneous application of an IgG-standard (human IgG1/kappa, Sigma #I3889). No unspecific background was detectable under these conditions as shown by use of medium supernatants of untransfected cells.

Example 4

Production of mAb425CH1-IL2 fusion protein

Generation of a tricistronic expression vector

Generation of the DNA sequence encoding the mAb425CH1-IL2 fusion protein has been disclosed in EP 0659 439 and EP 0706 799. A HindIII/EcoRI fragment containing the entire mAb425CH1-IL-2 heavy chain was ligated into the multi-cloning site of the pMC2PSΔH vector. The NotI/ClaI fragment of this construct was ligated with the ClaI/NotI fragment from pMCLΔHAP containing the mAb425 light chain. The resulting construct contains the light chain in the promoter-proximal position followed by the heavy-chain-IL-2 fusion and the Puromycin resistance. The genes are interspersed by two IRES sequences to allow transcription of all three cistrons into one messenger RNA.

Establishment of a recombinant BHK-21 cell line producing mAb425CH1-IL2 fusion protein

BHK-21 (ATCC CCL 10) were transfected with the tricistronic expression vector encoding mAb425CH1-IL2 fusion protein by the calcium phosphate method with a kit commercially available (InVitrogen) according to the manufacturer's instructions. Selection for transfected BHK-21A cells was done in the presence of 5 µg/ml Puromycin (Sigma). Clones are analyzed for expression of immunoconjugates by EGF-receptor or cytokine-specific ELISAs. Selected clones are then further purified by limiting dilution cloning. In the presence of Puromycin a lot of clones could be isolated which stably express the mAb425CH1-IL2 fusion protein. Three examples are shown in **Fig.** 2).

Example 5

Expression of a mAb425CH1-TNFα fusion protein

Generation of a tricistronic expression vector

Generation of the DNA sequence encoding the mAb425CH1-TNFα fusion protein has been disclosed in EP 0659 439 and EP 0706 799. The heavy chain-TNFα fusion gene construct was generated on the basis of the heavy chain-IL-2 fusion gene. The KpnI/EcoRI fragment containing part of the heavy chain variable region, the CH1 domain and IL-2 was subcloned into pUC19. In this construct the NcoI/EcoRI fragment containing the IL-2-encoding sequences was exchanged with the NcoI/EcoRI fragment containing the TNFα-encoding sequences. The KpnI/EcoRI fragment of this construct was combined in pUC18 with the HindIII/KpnI fragment containing the 5'part of the heavy chain variable region to generate the full length heavy chain-TNFα fusion gene. The HindIII/EcoRI fragment was ligated into the multi-cloning site of the pMC2PSΔH vector. The NotI/ClaI fragment of this construct was ligated with the ClaI/NotI fragment from pMCLΔHAP containing the mAb425 light chain. The resulting construct

contains the light chain in the promoter-proximal position followed by the heavy-chain-TNFα fusion and the Puromycin resistance. The genes are interspersed by two IRES sequences to allow transcription of all three cistrons into one messenger RNA.

Establishment of a recombinant BHK-21 cell line producing mAb425CH1-TNFα fusion protein

The establishment of a recombinant BHK-21 cell line producing mAb425CH1-TNFα fusion protein has been performed as described in example 5 for mAb425CH1-Il-2 fusion protein. We could isolate several clones which stably express the mAb425CH1-TNFα fusion protein for more than 12 weeks even without selection pressure. One example is shown in **Fig. 3**

Example 6

Expression of a mAb425CH3-IL-2 fusion protein

Generation of a tricistronic expression vector

Generation of the DNA sequence encoding the mAb425CH3-IL-2 fusion protein has been disclosed in EP 0659 439 and EP 0706 799. The HindIII/EcoRI fragment containing the complete heavy chain-IL-2 fusion gene was cloned into the multi-cloning vector pMC-2P. The NotI/ClaI fragment of this construct was ligated with the ClaI/NotI fragment from pMCLΔHAP containing the mAb425 light chain. The resulting construct contains the light chain in the promoter-proximal position followed by the heavy-chain-TNFα fusion and the Puromycin resistance. The genes are interspersed by two IRES sequences to allow transcription of all three cistrons into one messenger RNA.

Establishment of a recombinant BHK-21 cell line producing mAb425CH3-IL-2 fusion protein

Stable BHK-21 cell lines expressing mAb425CH3-IL-2 fusion protein have been established as described in example 5. Several clones could be isolated which stably express the mAb425CH3-IL-2 fusion protein for several weeks even in the absence of selection. One example is shown in **Fig. 4**

Purification of mAb425CH3-IL2

Transfected BHK cells (rBHK21A-CH3-IL2/K69-8) were fermented in a semicontinous manner and the fusion protein was isolated from the collected, cell free supernatant.

The first purification step was performed by affinity chromatography on carrier bound ProteinA (Pharmacia) using the extended bed technology. The starting conditions were 0,1 M phosphate buffer, pH 8,5. Impurities were removed with 0,2 M glycin buffer, pH 5,0 and subsequently, the fusion protein was eluted from the sedimented gel bed with 0,2 M glycin buffer, pH 3,3. The pH of the eluate was immediately neutralized by adding 10 % (vol./vol.) 1 M TRIS solution and brought up to pH 8 - 8,5.

In a second purification step further impurities were separated by cation exchange chromatography on Fractogel EMD SO_3^- 650(S) (Merck). The starting conditions were 10 mM phosphate buffer, pH 6,0 (conductivity 2 mS). The fusion protein was eluted with a NaCl-gradient 0 -0,6 M NaCl).

The final purification step was done by size exclusion chromatography on Fractogel BioSEC 650(S) (Merck) in PBS, pH 7,4. Up to 5 % aggregates and small amounts of impurities with smaller molecular weight were separated.

Concentration and diafiltration were done by ultrafiltration (Amicon). Membranes with a cut-off of 30 kDa were used.

Detection of protein-containing fractions was done by SDS-PAGE and an ELISA specific for human Ig with affinity-purified goat anti-human Fc as catcher antibody and affinity-purified goat anti human anti $F(ab)_2$ coupled to alkaline phospatase for detection (both Dianova).

The protein content of the preparation was about 1 mg/ml. The endotoxin content was < 1 EU/mg fusion protein. The purity of the protein preparation could be demonstrated by SDS Page **(Fig. 5)**. In Western Blots identity of heavy and light chain could be verified (data not shown).

Functional analysis of recombinant mAb425CH3-IL-2 fusion protein

FACS analysis with EGF-R-positive cells showed that binding of the antibody portion is identical to a mAb425 control **(Fig. 6)**. Furthermore, IL-2 activity is indistinguishable from the activity of recombinant IL-2 **(Fig. 7)**, indicating that interaction of the fusion protein with the IL-2 receptor is not impaired in the fusion protein. Taken together, it can be concluded that the expression system described herein provides high amounts of the mAb425CH3-IL-2 fusion protein which is fully active with respect to antigen binding and IL-2 activity.

Example 7

Expression of a mAb425CH3-TNFα fusion protein

Generation of a tricistronic expression vector

The PCR amplified coding region of the recombinant light chain (HindIII-EcoRI) gene was inserted into pMC-1 at the polylinker site. The puromycin resistance gene coding sequence was inserted between the IRES sequence and the polyadenylation site of pMC-2 to give pMC-2P. The heavy chain-cytokine fusion protein genes were inserted into the polylinker sequence of pMC-2P. The XmnI/NotI fragments of both Immunoglobulin chain vectors were combined to give e.g. pMCLDHAP, a 8298 bp tricistronic expression vector for IgG-TNF-alpha and puromycin acetyltransferase **(Fig. 8)**.

Establishment of a recombinant BHK-21 cell line producing mAb425CH3-TNFα fusion protein

BHK-21 cells were transfected with the tricistronic expression vector encoding mAb425CH3-TNFα fusion protein using the calcium phosphate precipitation method as detailed by Mielke et al. (1990, Biochemistry

29:7475). 5 µg of uncut plasmid were used without the addition of carrier DNA. Stable transfectants were selected and cultivated in medium containing Puromycin (Sigma) at a final concentration of 5 µg/ml. Clones are analysed for expression of immunoconjugates by IgG-specific ELISA. Selected clones were further purified by limiting dilution cloning. We could isolate several clones which stably express mAb425CH3-TNFα fusion protein even in the absence of selection. One example is shown in **Fig. 9**.

Chromosomal DNA analysis

Isolation of genomic DNA: Cells from a 141 cm^2 culture dish were harvested in 20 ml TEN buffer [40mM Tris/HC1 (pH 7.5), 1mM EDTA, 150 mM NaCL], split into two portions and pelleted for 5 min at 1000 rpm in a table top centrifuge. One of these cell pellets was intensively resuspended in 1 ml of TEN and then provided with 1ml of 2x extraction buffer [20mM tris/HCl (pH 8), 200 mM EDTA, 1 % SDS, 40 µg/ml Rnase A] . After 5 h of incubation at 37 ° C, 50 µl Proteinase K solution (20 mg/ml) was added and incubation was continued over night. Following a standard phenolization step, the DNA solution was dialyzed against TE and was then used without any further precipitation steps.

Southern Blots/Methylation pattern: 20µg of genomic DNA was digested over night with the indicated restriction enzyme in a total volume of 500µl, precipitated by addition of 300 µl 2-propanol and pelleted at 13000 rpm, 4 ° C in a microcentrifuge. DNA pellets were carefully resuspended in 40µl of 1x loading buffer [2.5 % Ficoll (Type 400), 17 mM EDTA, o.o1 % Xylene Cyanol FF), 20µl were applied on a 0.8 % TAE agarose gel and electrophoresed. Gels were then blotted onto nylon membranes (Zeta probe, Biorad) with 0.4 M NaOH over night and membranes were then hybridized to the indicated radiolabelled (Rediprime, Amersham) DNA probes according to manufacturers recommendations and following the protocol of Church and Gilbert (Church, G.M. and Gilbert, W. (1984), PNAS 81, 1991 - 1995). **(Fig.10)**

Purification of mAb425CH3-TNFα

Transformed BHK cells (rBHK21A-CH3-TNFα/SC7.4) were fermented in a semicontinous manner and the fusion protein was isolated from the collected, cell free supernatant.

The first purification step performed by affinity chromatography on carrier bound ProteinA (Pharmacia) using the extended bed technology. The starting conditions were 0,1 M phosphate buffer, pH 8,5. Impurities were removed with 0,2 M glycin buffer, pH 5,0 before the fusion protein was eluted from the sedimented gel bed with 0,2 M glycin buffer, pH 3,3. The pH of the eluat was immediately brought up to pH 8 - 8,5 by adding 10 % (vol./vol.) 1 M TRIS solution.

The second purification step was done by chromatography on hydroxyapatite (Merck). The starting conditions were 5 mM phosphate, pH 7,0. The elution was performed with a phosphate gradient (5 - 500 mM).

The final purification step was done by size exclusion chromatography on Fractogel BioSEC 650(S) (Merck) in PBS, pH 7,4 as described above. Up to 5 % aggregates and small amounts of impurities with smaller molecular weight were separated.

Concentration and diafiltration were done by ultrafiltration. Membranes with a cut-off of 30 kDa were used.

Detection of protein-containing fractions was done by SDS-PAGE and an ELISA specific for human Ig with affinity-purified goat anti-human Fc as catcher antibody and affinity-purified goat anti human anti $F(ab)_2$ coupled to alkaline phospatase for detection (both Dianova).

The protein content of the preparation was about 1 mg/ml. The endotoxin content was < 1 EU/mg fusion protein.

Assessment of functionality of mAb425CH3-TNFα fusion protein

The functionality of mAb425CH3-TNFα with respect to antigen binding was demonstrated by FACS analysis **(Fig. 11)**. The fusion protein does bind to EGF-R-positive cells with the same quality as the mAb425 control antibody.

TNFa activity of the mAb425CH3-TNFα fusion protein was investigated on different human tumor cell lines. MCF7 is a human mamma carcinoma cell line which is not EGF-R positive. The inhibition of proliferation is therefore exclusively based on TNFα activity. As demonstrated in **Fig. 12** the growth inhibition induced by the mAb425CH3-TNFα fusion protein is virtually identical to that of recombinant TNFα. mAb425 alone does not have any effect on proliferation of MCF7.

mAb425 was raised against the human carcinoma cell line A431 which is highly positive for EGF-R expression (Rodeck et al.). It was demonstrated previously that mAb425 is internalized upon binding to A431 cells. A431 is not TNFα sensitive and neither mAb425CH3-TNFα fusion protein nor the combination of mAb425 and recombinant TNFα does have any effect on the growth of A431 cells **(Fig. 13)** indicating that the growth inhibition specifically requires expression of TNFα receptors. Lack of TNFα receptors cannot be overcome through internalization of mAb425CH3-TNFα fusion protein mediated by EGF-R receptor.

BT20, a human mamma carcinoma cell line and C8161, a human melanoma cell line, are both EGF-R positive and TNFα sensitive. The density of EGF-R on the cell surface is higher on BT20 than on C8161 as determined by FACS analysis (data not shown). The proliferation of both cell lines is strongly inhibited by mAb425CH3-TNFα fusion protein **(Fig. 14)**. The effect is more pronounced on BT20 cells than on C8161, which might be due to the increased EGF-R expression which leads to a higher crosslinking of TNFα receptors and thus increased signal transduction. These experiments clearly demonstrate the superiority of the mAb425CH3-TNFα fusion protein when compared to the combination of mAb425 and TNFα. This could be explained by the crosslinking of TNFα receptors on one side due to capping of EGF-R on the other side. Thereby signal transduction is maximally enhanced.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Merck Patent GmbH
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(F) POSTAL CODE (ZIP): 64271
(G) TELEPHONE: 49-6151-72-7022
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(ii) TITLE OF INVENTION: Oligocistronic Expression System for the Production of Antibody Fusion Proteins

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8298 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: humanized mAb425-TNFalpha Fusion protein
(B) STRAIN: E. coli K12
(G) CELL TYPE: Fibroblast
(H) CELL LINE: BHK-21

(ix) FEATURE:
(A) NAME/KEY: promoter
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(D) OTHER INFORMATION:/function= "Enhancer/promoter: MPSV/CMV"

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(B) LOCATION:905..976

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(B) LOCATION:5877..8298
(D) OTHER INFORMATION:/product= "DNA sequence comprising SV40 PolyA (5929-6181)"
/standard_name= "SV40 PolyA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GAAACAGGAG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG CCCCGCTCAG 180

GGCCAAGAAC AGTTGGAACA GGAGAATTGG GCCAAACAGG ATATCTGTGG TAAGCAGTTC 240

CTGCCCCGCT CAGGGCCAAG AACAGATGGT CCCCAGATGC GGTCCCGCCC TCAGCAGTTT 300

CTAGACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT 360

GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA 420

ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC 480

AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA 540

CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC 600

CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG 660

ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC AAAATCAACG 720

GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT 780

ACGGTGGGAG GTCTATATAA GCAGAGCTCG TTTAGTGAAC CGTCAGATCG CCTGGAGACG 840

CCATCCACGC TGTTTTGACC TCCATAGAAG ACACCGGGAC CGATCCAGCC TCGAGGAACT 900

GGAAAACCAG AAAGTTAACT GGTAAGTTTA GTCTTTTTGT CTTTTATTTC AGGTCCCGGA 960

ATTAAGCTTC GCCACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA 1009
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val
1 5 10

GCA ACA GCT ACAGGTAAGG GGCTCACAGT AGCAGGCTTG AGGTCTGGAC 1058
Ala Thr Ala

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ATATATATGG GTGACAATGA CATCCACTTT GCCTTTCTCT CCACAGGT GTC CAC TCC      1115
                                                    Val His Ser
                                                      1

GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT      1163
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
  5                  10                  15

GAC AGA GTG ACC ATC ACC TGT AGT GCC AGC TCA AGT GTA ACT TAC ATG      1211
Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val Thr Tyr Met
 20                  25                  30                  35

TAT TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC      1259
Tyr Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr
             40                  45                  50

GAC ACA TCC AAC CTG GCT TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC      1307
Asp Thr Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
         55                  60                  65

GGT AGC GGT ACC GAC TAC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG      1355
Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu
     70                  75                  80

GAC ATC GCC ACC TAC TAC TGC CAG CAG TGG AGT AGT CAC ATA TTC ACG      1403
Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser His Ile Phe Thr
  85                  90                  95

TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGTGAGTAGA ATTTAAACTT       1453
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100                 105

TGCTTCCTCA GTTGGATCCA TCTGGGATAA GCATGCTGTT TTCTGTCTGT CCCTAACATG   1513

CCCTGTGATT ATGCGCAAAC AACACACCCA AGGGCAGAAC TTTGTTACTT AAACACCATC   1573

CTGTTTGCTT CTTTCCTCAG GA ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC     1625
                        Thr Val Ala Ala Pro Ser Val Phe Ile Phe
                          1               5                  10

CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC      1673
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys
                 15                  20                  25

CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG AAG GTG      1721
Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
             30                  35                  40

GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC ACA GAG CAG      1769
Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln
         45                  50                  55
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GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG ACG CTG AGC      1817
Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser
     60                  65                  70

AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT      1865
Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His
 75                  80                  85                  90

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG TGT      1913
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
                95                 100                 105

TAGAATTCAG CTTTTAAAAC AGCTCTGGGG TTGTACCCAC CCCAGAGGCC CACGTGGCGG    1973

CTAGTACTCC GGTATTGCGG TACCCTTGTA CGCCTGTTTT ATACTCCCTT CCCGTAACTT    2033

AGACGCACAA AACCAAGTTC AATAGAAGGG GGTACAAACC AGTACCACCA CGAACAAGCA    2093

CTTCTGTTTC CCCGGTGATG TCGTATAGAC TGCTTGCGTG GTTGAAAGCG ACGGATCCGT    2153

TATCCGCTTA TGTACTTCGA GAAGCCCAGT ACCACCTCGG AATCTTCGAT GCGTTGCGCT    2213

CAGCACTCAA CCCCAGAGTG TAGCTTAGGC TGATGAGTCT GGACATCCCT CACCGGTGAC    2273

GGTGGTCCAG GCTGCGTTGG CGGCCTACCT ATGGCTAACG CCATGGGACG CTAGTTGTGA    2333

ACAAGGTGTG AAGAGCCTAT TGAGCTACAT AAGAATCCTC CGGCCCCTGA ATGCGGCTAA    2393

TCCCAACCTC GGAGCAGGTG GTCACAAACC AGTGATTGGC CTGTCGTAAC GCGCAAGTCC    2453

GTGGCGGAAC CGACTACTTT GGGTGTCCGT GTTTCCTTTT ATTTTATTGT GGCTGCTTAT    2513

GGTGACAATC ACAGATTGTT ATCATAAAGC GAATTGGATT GCGGCCGCGA ATTAAGCTTG    2573

CCGCCACC ATG GAC TGG ACC TGG CGC GTG TTT TGC CTG CTC GCC GTG GCT      2623
         Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala
           1               5                  10

CCT GGG GCC CAC AGC CAG GTG CAA CTA GTG CAG TCC GGC GCC GAA GTG      2671
Pro Gly Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val
 15                  20                  25                  30

AAG AAA CCC GGT GCT TCC GTG AAG GTG AGC TGT AAA GCT AGC GGT TAT      2719
Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr
                35                  40                  45

ACC TTC ACA TCC CAC TGG ATG CAT TGG GTT AGA CAG GCC CCA GGC CAA      2767
Thr Phe Thr Ser His Trp Met His Trp Val Arg Gln Ala Pro Gly Gln
             50                  55                  60

GGG CTC GAG TGG ATT GGC GAG TTC AAC CCT TCA AAT GGC CGG ACA AAT      2815
Gly Leu Glu Trp Ile Gly Glu Phe Asn Pro Ser Asn Gly Arg Thr Asn
         65                  70                  75
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TAT AAC GAG AAG TTT AAG AGC AAG GCT ACC ATG ACC GTG GAC ACC TCT      2863
Tyr Asn Glu Lys Phe Lys Ser Lys Ala Thr Met Thr Val Asp Thr Ser
     80                  85                  90

ACA AAC ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG GAC ACT      2911
Thr Asn Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
 95                 100                 105                 110

GCA GTC TAC TAC TGC GCC TCA CGG GAT TAC GAT TAC GAT GGC AGA TAC      2959
Ala Val Tyr Tyr Cys Ala Ser Arg Asp Tyr Asp Tyr Asp Gly Arg Tyr
                115                 120                 125

TTC GAC TAT TGG GGA CAG GGT ACC CTT GTC ACC GTC AGT TCA GGT GAG      3007
Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Gly Glu
            130                 135                 140

TGG ATC CTC TGC GCC TGG GCC CAG CTC TGT CCC ACA CCG CGG TCA CAT      3055
Trp Ile Leu Cys Ala Trp Ala Gln Leu Cys Pro Thr Pro Arg Ser His
        145                 150                 155

GGC ACC ACC TCT CTT GCA GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC      3103
Gly Thr Thr Ser Leu Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro
    160                 165                 170

CTG GCA CCC TCC TCC AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC      3151
Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
175                 180                 185                 190

TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC      3199
Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn
                195                 200                 205

TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG      3247
Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln
            210                 215                 220

TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC      3295
Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser
        225                 230                 235

AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC      3343
Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser
    240                 245                 250

AAC ACC AAG GTG GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT      3391
Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr
255                 260                 265                 270

CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA      3439
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser
                275                 280                 285
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GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG      3487
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
            290                 295                 300

ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT      3535
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro
        305                 310                 315

GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC      3583
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
    320                 325                 330

AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGG GTG GTC      3631
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val
335                 340                 345                 350

AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC      3679
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
                355                 360                 365

AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC      3727
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
            370                 375                 380

ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG      3775
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
        385                 390                 395

CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC      3823
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
    400                 405                 410

CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC      3871
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
415                 420                 425                 430

AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC      3919
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
                435                 440                 445

TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC      3967
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
            450                 455                 460

AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT      4015
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
        465                 470                 475

CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA      4063
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    480                 485                 490
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ATG GTC AGA TCA TCT TCG CGA ACC CCG AGT GAC AAG CCT GTA GCC CAT      4111
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
495                 500                 505                 510

GTT GTA GCA AAC CCT CAA GCT GAG GGG CAA CTG CAG TGG CTG AAC CGC      4159
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
                515                 520                 525

CGG GCC AAT GCC CTC CTG GCC AAT GGC GTC GAG CTG AGA GAT AAC CAG      4207
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
            530                 535                 540

CTG GTG GTG CCA TCA GAG GGC CTG TAC CTC ATC TAC TCC CAG GTC CTC      4255
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
        545                 550                 555

TTC AAG GGC CAA GGC TGC CCG TCG ACC CAT GTG CTC CTC ACC CAC ACC      4303
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
    560                 565                 570

ATC AGC CGC ATC GCC GTC TCC TAC CAG ACC AAG GTT AAC CTC CTC TCT      4351
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
575                 580                 585                 590

GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT GAG GCC      4399
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
                595                 600                 605

AAG CCC TGG TAT GAG CCC ATC TAT CTG GGA GGG GTC TTC CAG CTC GAG      4447
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
            610                 615                 620

AAG GGT GAC CGA CTC AGC GCT GAG ATC AAT CGG CCC GAC TAT CTC GAC      4495
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
        625                 630                 635

TTT GCC GAG TCC GGA CAG GTC TAC TTT GGG ATC ATT GCC CTG              4537
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
    640                 645                 650

TGATAAGGAT CCCCGGGTAC CGAGCTCGAA TTCAGCTTTT AAAACAGCTC TGGGGTTGTA    4597

CCCACCCCAG AGGCCCACGT GGCGGCTAGT ACTCCGGTAT TGCGGTACCC TTGTACGCCT    4657

GTTTTATACT CCCTTCCCGT AACTTAGACG CACAAAACCA AGTTCAATAG AAGGGGGTAC    4717

AAACCAGTAC CACCACGAAC AAGCACTTCT GTTTCCCCGG TGATGTCGTA TAGACTGCTT    4777

GCGTGGTTGA AAGCGACGGA TCCGTTATCC GCTTATGTAC TTCGAGAAGC CCAGTACCAC    4837

CTCGGAATCT TCGATGCGTT GCGCTCAGCA CTCAACCCCA GAGTGTAGCT TAGGCTGATG    4897

AGTCTGGACA TCCCTCACCG GTGACGGTGG TCCAGGCTGC GTTGGCGGCC TACCTATGGC    4957
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TAACGCCATG GGACGCTAGT TGTGAACAAG GTGTGAAGAG CCTATTGAGC TACATAAGAA      5017

TCCTCCGGCC CCTGAATGCG GCTAATCCCA ACCTCGGAGC AGGTGGTCAC AAACCAGTGA      5077

TTGGCCTGTC GTAACGCGCA AGTCCGTGGC GGAACCGACT ACTTTGGGTG TCCGTGTTTC      5137

CTTTTATTTT ATTGTGGCTG CTTATGGTGA CAATCACAGA TTGTTATCAT AAAGCGAATT      5197

GGATTGCGGC CGGCCGCCAC GACCGGTGCC GCCACCATCC CCTGACCCAC GCCCCTGACC      5257

CCTCACAAGG AGACGACCTT CC ATG ACC GAG TAC AAG CCC ACG GTG CGC CTC       5309
                         Met Thr Glu Tyr Lys Pro Thr Val Arg Leu
                           1               5                  10

GCC ACC CGC GAC GAC GTC CCC CGG GCC GTA CGC ACC CTC GCC GCC GCG       5357
Ala Thr Arg Asp Asp Val Pro Arg Ala Val Arg Thr Leu Ala Ala Ala
                  15                  20                  25

TTC GCC GAC TAC CCC GCC ACG CGC CAC ACC GTC GAC CCG GAC CGC CAC      5405
Phe Ala Asp Tyr Pro Ala Thr Arg His Thr Val Asp Pro Asp Arg His
              30                  35                  40

ATC GAG CGG GTC ACC GAG CTG CAA GAA CTC TTC CTC ACG CGC GTC GGG       5453
Ile Glu Arg Val Thr Glu Leu Gln Glu Leu Phe Leu Thr Arg Val Gly
          45                  50                  55

CTC GAC ATC GGC AAG GTG TGG GTC GCG GAC GAC GGC GCC GCG GTG GCG       5501
Leu Asp Ile Gly Lys Val Trp Val Ala Asp Asp Gly Ala Ala Val Ala
      60                  65                  70

GTC TGG ACC ACG CCG GAG AGC GTC GAA GCG GGG GCG GTG TTC GCC GAG       5549
Val Trp Thr Thr Pro Glu Ser Val Glu Ala Gly Ala Val Phe Ala Glu
  75                  80                  85                  90

ATC GGC CCG CGC ATG GCC GAG TTG AGC GGT TCC CGG CTG GCC GCG CAG       5597
Ile Gly Pro Arg Met Ala Glu Leu Ser Gly Ser Arg Leu Ala Ala Gln
                  95                 100                 105

CAA CAG ATG GAA GGC CTC CTG GCG CCG CAC CGG CCC AAG GAG CCC GCG       5645
Gln Gln Met Glu Gly Leu Leu Ala Pro His Arg Pro Lys Glu Pro Ala
              110                 115                 120

TGG TTC CTG GCC ACC GTC GGC GTC TCG CCC GAC CAC CAG GGC AAG GGT       5693
Trp Phe Leu Ala Thr Val Gly Val Ser Pro Asp His Gln Gly Lys Gly
          125                 130                 135

CTG GGC AGC GCC GTC GTG CTC CCC GGA GTG GAG GCG GCC GAG CGC GCC       5741
Leu Gly Ser Ala Val Val Leu Pro Gly Val Glu Ala Ala Glu Arg Ala
      140                 145                 150
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GGG GTG CCC GCC TTC CTG GAG ACC TCC GCG CCC CGC AAC CTC CCC TTC 5789
Gly Val Pro Ala Phe Leu Glu Thr Ser Ala Pro Arg Asn Leu Pro Phe
155 160 165 170

TAC GAG CGG CTC GGC TTC ACC GTC ACC GCC GAC GTC GAG TGC CCG AAG 5837
Tyr Glu Arg Leu Gly Phe Thr Val Thr Ala Asp Val Glu Cys Pro Lys
175 180 185

GAC CGC GCG ACC TGG TGC ATG ACC CGC AAG CCC GGT GCC TGACGCCCGC 5886
Asp Arg Ala Thr Trp Cys Met Thr Arg Lys Pro Gly Ala
190 195

CCCACGACCC GCAGCGCCCG ACCGAAAGGA GCGCACGACC CCATGAGCTT CGATCCAGAC 5946

ATGATAAGAT ACATTGATGA GTTTGGACAA ACCACAACTA GAATGCAGTG AAAAAAATGC 6006

TTTATTTGTG AAATTTGTGA TGCTATTGCT TTATTTGTAA CCATTATAAG CTGCAATAAA 6066

CAAGTTAACA ACAACAATTG CATTCATTTT ATGTTTCAGG TTCAGGGGGA GGTGTGGGAG 6126

GTTTTTTAAA GCAAGTAAAA CCTCTACAAA TGTGGTATGG CTGATTATGA TCCTGCCTCG 6186

CGCGTTTCGG TGATGACGGT GAAAACCTCT GACACATGCA GCTCCCGGAG ACGGTCACAG 6246

CTTGTCTGTA AGCGGATGCC GGGAGCAGAC AAGCCCGTCA GGGCGCGTCA GCGGGTGTTG 6306

GCGGGTGTCG GGGCGCAGCC ATGACCCAGT CACGTAGCGA TAGCGGAGTG TATACTGGCT 6366

TAACTATGCG GCATCAGAGC AGATTGTACT GAGAGTGCAC CATATGTCGG GCCGCGTTGC 6426

TGGCGTTTTT CCATAGGCTC CGCCCCCCTG ACGAGCATCA CAAAAATCGA CGCTCAAGTC 6486

AGAGGTGGCG AAACCCGACA GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC 6546

TCGTGCGCTC TCCTGTTCCG ACCCTGCCGC TTACCGGATA CCTGTCCGCC TTTCTCCCTT 6606

CGGGAAGCGT GGCGCTTTCT CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG 6666

TTCGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT 6726

CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA CTGGCAGCAG 6786

CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT 6846

GGTGGCCTAA CTACGGCTAC ACTAGAAGGA CAGTATTTGG TATCTGCGCT CTGCTGAAGC 6906

CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA 6966

GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG AAAAAAAGGA TCTCAAGAAG 7026

ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA CGTTAAGGGA 7086

TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA 7146

GTTTTAAATC AATCTAAAGT ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA 7206

TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT GCCTGACTCC 7266

CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA 7326

TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG CCAGCCGGAA 7386

GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT 7446

GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG TTAATAGTGC GCAACGTTGT TGCCATTGCT 7506

ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC CGGTTCCCAA 7566

CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT 7626

CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA 7686

CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC TGGTGAGTAC 7746

TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA 7806

ACACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT 7866

TCTTCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC 7926

ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA 7986

AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA 8046

CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC 8106

GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC 8166

CGAAAAGTGC CACCTGACGT CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT 8226

AGGCGTATCA CGAGGCCCTT TCGTCTTCAA GAATTGGTCG ATCGACCAAT TCTCATGTTT 8286

GACAGCTTAT CA 8298

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala

1 5 10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
1 5 10 15

Ser Val Gly Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Ser Ser Val
20 25 30

Thr Tyr Met Tyr Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu
35 40 45

Leu Ile Tyr Asp Thr Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe
50 55 60

Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu
65 70 75 80

Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Ser His
85 90 95

Ile Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln
1 5 10 15

Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr
20 25 30

Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser
35 40 45

Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
50 55 60

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
65 70 75 80

His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro
85 90 95

Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
100 105

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 652 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Asp Trp Thr Trp Arg Val Phe Cys Leu Leu Ala Val Ala Pro Gly
1 5 10 15

Ala His Ser Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30

Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe
35 40 45

Thr Ser His Trp Met His Trp Val Arg Gln Ala Pro Gly Gln Gly Leu
50 55 60

Glu Trp Ile Gly Glu Phe Asn Pro Ser Asn Gly Arg Thr Asn Tyr Asn
65 70 75 80

Glu Lys Phe Lys Ser Lys Ala Thr Met Thr Val Asp Thr Ser Thr Asn
85 90 95

Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val
100 105 110

Tyr Tyr Cys Ala Ser Arg Asp Tyr Asp Tyr Asp Gly Arg Tyr Phe Asp
115 120 125

Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Gly Glu Trp Ile
130 135 140

Leu Cys Ala Trp Ala Gln Leu Cys Pro Thr Pro Arg Ser His Gly Thr
145 150 155 160

Thr Ser Leu Ala Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala
165 170 175

Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu
180 185 190

Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly
195 200 205

Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser
210 215 220

Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu
225 230 235 240

Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr
245 250 255

Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
260 265 270

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
275 280 285

Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
290 295 300

Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
305 310 315 320

Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
325 330 335

Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
340 345 350

Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
355 360 365

Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
370 375 380

Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
385 390 395 400

Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
405 410 415

Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
420 425 430

Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
435 440 445

Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
450 455 460

Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
465 470 475 480

Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Met Val
485 490 495

Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val
500 505 510

Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala
515 520 525

Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val
530 535 540

Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys
545 550 555 560

Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile Ser
565 570 575

Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile
580 585 590

Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro
595 600 605

Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly
610 615 620

Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala
625 630 635 640

Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
645 650

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 199 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Thr Glu Tyr Lys Pro Thr Val Arg Leu Ala Thr Arg Asp Asp Val
1 5 10 15

Pro Arg Ala Val Arg Thr Leu Ala Ala Ala Phe Ala Asp Tyr Pro Ala
20 25 30

Thr Arg His Thr Val Asp Pro Asp Arg His Ile Glu Arg Val Thr Glu
35 40 45

Leu Gln Glu Leu Phe Leu Thr Arg Val Gly Leu Asp Ile Gly Lys Val
50 55 60

Trp Val Ala Asp Asp Gly Ala Ala Val Ala Val Trp Thr Thr Pro Glu
65 70 75 80

Ser Val Glu Ala Gly Ala Val Phe Ala Glu Ile Gly Pro Arg Met Ala
85 90 95

Glu Leu Ser Gly Ser Arg Leu Ala Ala Gln Gln Gln Met Glu Gly Leu
100 105 110

Leu Ala Pro His Arg Pro Lys Glu Pro Ala Trp Phe Leu Ala Thr Val
115 120 125

Gly Val Ser Pro Asp His Gln Gly Lys Gly Leu Gly Ser Ala Val Val
130 135 140

Leu Pro Gly Val Glu Ala Ala Glu Arg Ala Gly Val Pro Ala Phe Leu
145 150 155 160

Glu Thr Ser Ala Pro Arg Asn Leu Pro Phe Tyr Glu Arg Leu Gly Phe
165 170 175

Thr Val Thr Ala Asp Val Glu Cys Pro Lys Asp Arg Ala Thr Trp Cys
180 185 190

Met Thr Arg Lys Pro Gly Ala
195

Patent Claims

1. Oligocistronic expression vector suitable for the production of a heteromeric protein consisting of at least two protein chains in a mammalian host cell comprising
   (i) a promoter / enhancer sequence,
   (ii) a sequence encoding a first chain of the heteromeric protein or a fragment thereof,
   (iii) a sequence encoding a second chain of the heteromeric protein or a fragment thereof,
   (iv) optionally a sequence encoding a third or further chain of the heteromeric protein or a fragment thereof,
   (v) a sequence encoding a selection marker, and
   (vi) at least two sequences comprising a 5'-UTR poliovirus sequence containing an IRES element.

2. Expression vector according to claim 1, wherein the sequences (i) to (vi) are in the following order from upstream to downstream progression of said vector construct:
   (1) a sequence comprising the promoter / enhancer sequence (i),
   (2) sequence comprising the sequence encoding a first chain of the heteromeric protein or a fragment thereof (ii),
   (3) a sequence (vi) comprising a first IRES element,
   (4) a sequence comprising the sequence encoding a second chain of the heteromeric protein or a fragment thereof (iii),
   (5) a sequence (vi) comprising a second IRES element,
   (6) optionally a sequence comprising the sequence encoding a third or chain of the heteromeric protein or a fragment thereof (iv), and a sequence comprising a third or further IRES element (vi) located behind

the third or further sequence encoding the corresponding chain,

(7) a sequence comprising the selection marker (v).

3. Tricistronic expression vector according to claim 1 or 2 (comprising two IRES elements) wherein the sequence (ii) encodes the light chain and the sequence (iii) comprises a sequence encoding the heavy chain of a monoclaonal antibody (iiia), and sequences (iv) are not present.

4. Tricistronic expression vector according to claim 3, wherein the sequence (iii) comprises besides sequence (iiia) a sequence (iiib) encoding a biologically active ligand in order to produce an antibody fusion protein.

5. Expression vector according to claims 3 to 4 wherein the sequence (iiia) is shortend at its C-terminus and the sequence (iiib) is shortened at its N-terminus by a number of nucleotides each coding for 1 to 20 amino acids.

6. Expression vector according to claims 3 to 5, wherein a sequence (iiib) is used encoding a cytokine or chemokine.

7. Expression vector according to claim 6, wherein a sequence (iiib) is used encoding TNF alpha or IL-2.

8. Expression vector according to claim 1 to 7, wherein sequences (ii) and (iii) encoding the light and heavy chain of a monoclonal anti-EGFR antibody are used.

9. Expression vector according to claim 8 comprising the sequences encoding humanized monoclonal antibody 425 (mAb425).

10.Expression vector according to claim 3 comprising the CMV/MPSV promoter/enhancer sequence followed by the sequence encoding the mAb425 light chain, followed by the sequence from 5' UTR poliovirus containing an IRES element, followed by a fusion gene encoding a fusion protein consisting of the heavy chain of humanized mAb425 and fused at its C-terminus the sequence encoding TNF alpha or IL-2, followed by another IRES element from 5' UTR poliovirus, followed by a sequence coding for puromycin acetyl transferase and, finally the sequence of the polyadenylation signal of SV40.

11.Expression vector according to claim 10 comprising the DNA sequence which codes for the amino acid each depicted in Fig. 15.

12.Expression vector according to claims 1 to 10, comprising, additionally, two SAR elements.

13.Expression system comprising a mammalian host cell transformed with an expression vector specified in one of the claims 1 to 12.

14.Expression system acccording to claim 13, wherein the host cell is CHO, BHK-21 or SP2/0.

15.Process for the production of a heteomeric protein or fragments thereof by cultivating the host cells of an expression system specified in claim 13 in a suitable nutrient and separating the complete and active heteromeric protein from the cells and / or the medium.

16.Process according to claim 15 for the production of mAb425/TNF-alpha or mAb425/Il-2 Antibody fusion proteins or fragments thereof.

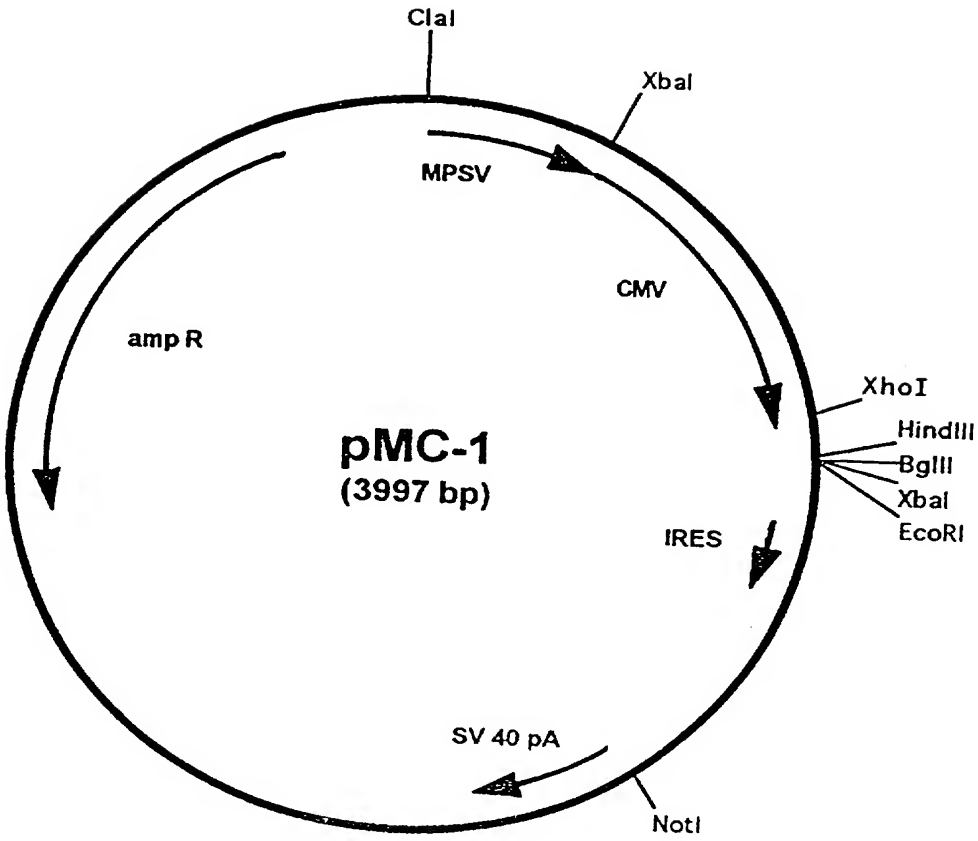

ClaI
XbaI
MPSV
CMV
amp R
XhoI
HindIII
BglII
XbaI
EcoRI
pMC-1
(3997 bp)
IRES
SV 40 pA
NotI

FIG. 1 A

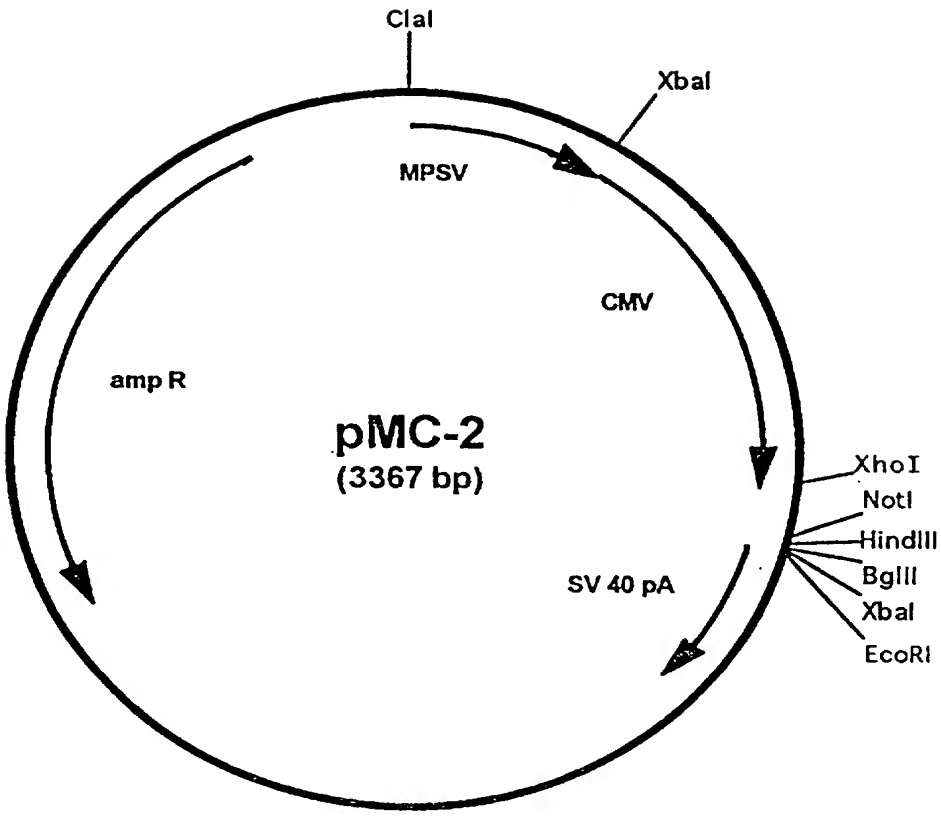

ClaI
XbaI
MPSV
CMV
amp R
pMC-2
(3367 bp)
XhoI
NotI
HindIII
BglII
XbaI
EcoRI
SV 40 pA

FIG. 1 B

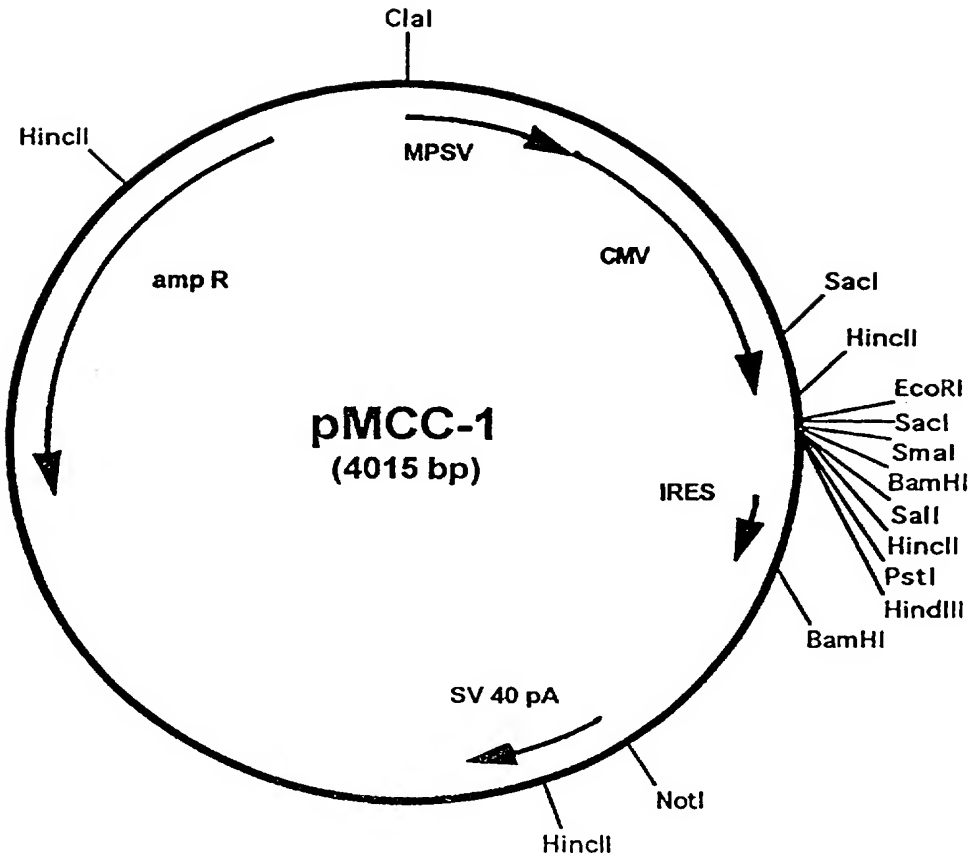

ClaI
HincII
MPSV
amp R
CMV
SacI
HincII
EcoRI
SacI
SmaI
BamHI
SalI
HincII
PstI
HindIII
BamHI
pMCC-1
(4015 bp)
IRES
SV 40 pA
NotI
HincII

FIG. 1 C

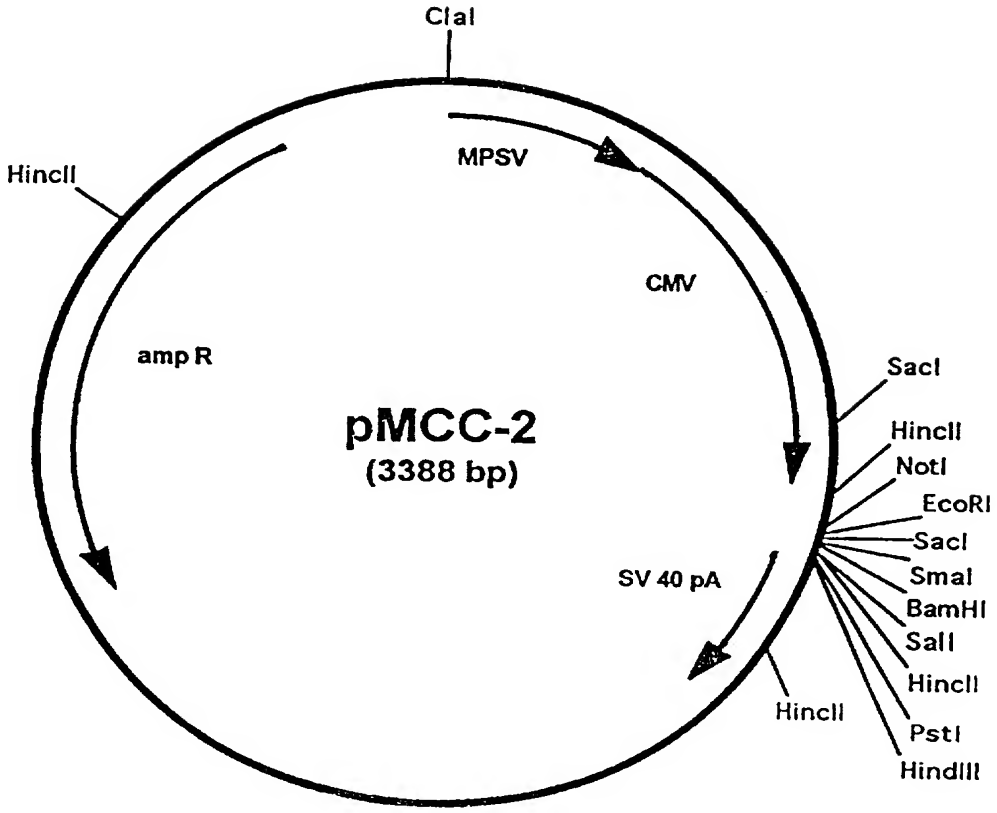

ClaI
MPSV
HincII
CMV
amp R
SacI
pMCC-2
(3388 bp)
HincII
NotI
EcoRI
SacI
SmaI
BamHI
SalI
HincII
PstI
HindIII
SV 40 pA
HincII

FIG. 1 D

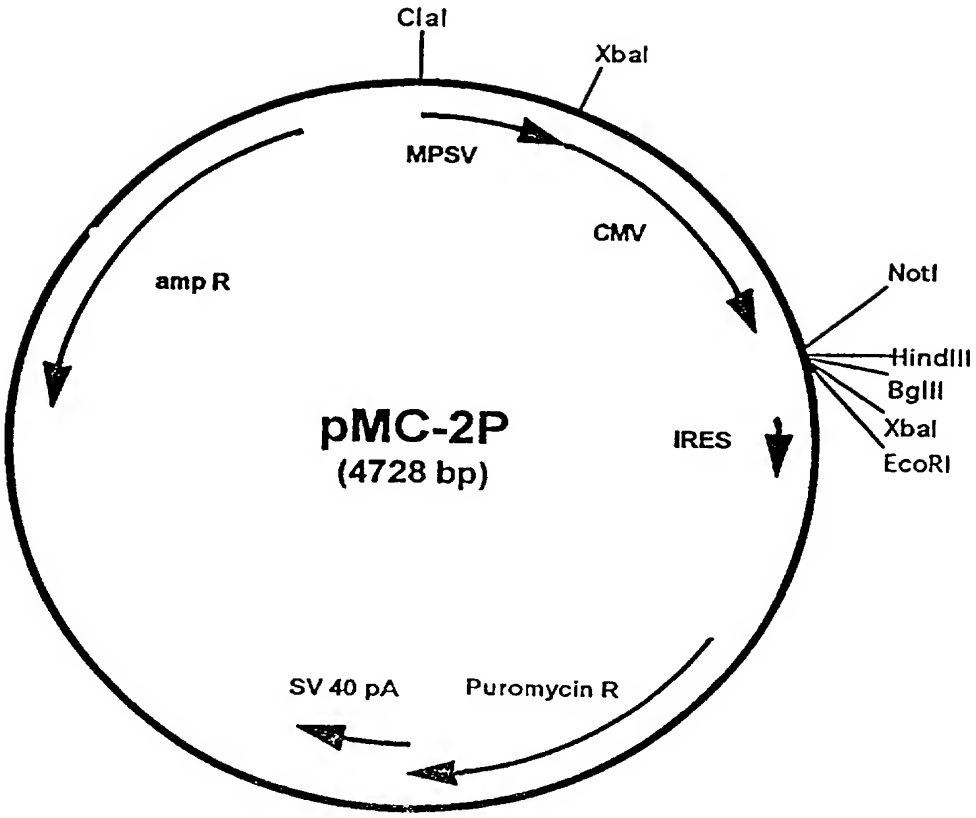

ClaI
XbaI
MPSV
CMV
amp R
NotI
HindIII
BglII
XbaI
EcoRI
pMC-2P
(4728 bp)
IRES
SV 40 pA
Puromycin R

FIG. 1 E

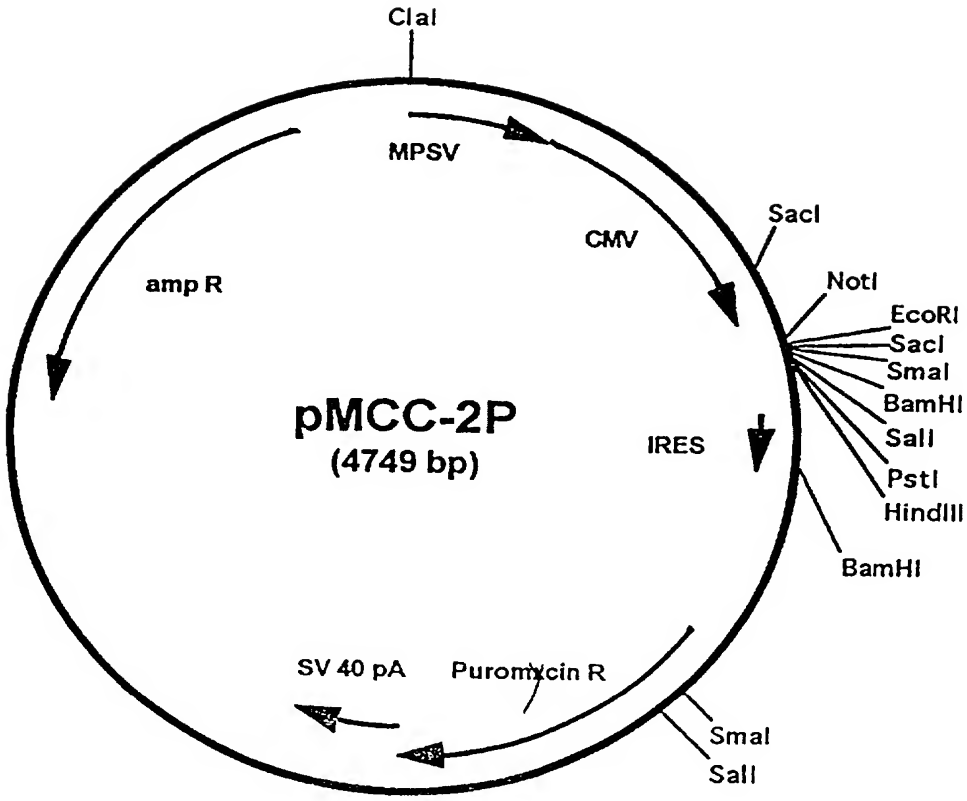

ClaI
MPSV
CMV
SacI
NotI
EcoRI
SacI
SmaI
BamHI
SalI
PstI
HindIII
BamHI
amp R
pMCC-2P
(4749 bp)
IRES
SV 40 pA
Puromycin R
SmaI
SalI

FIG. 1 F

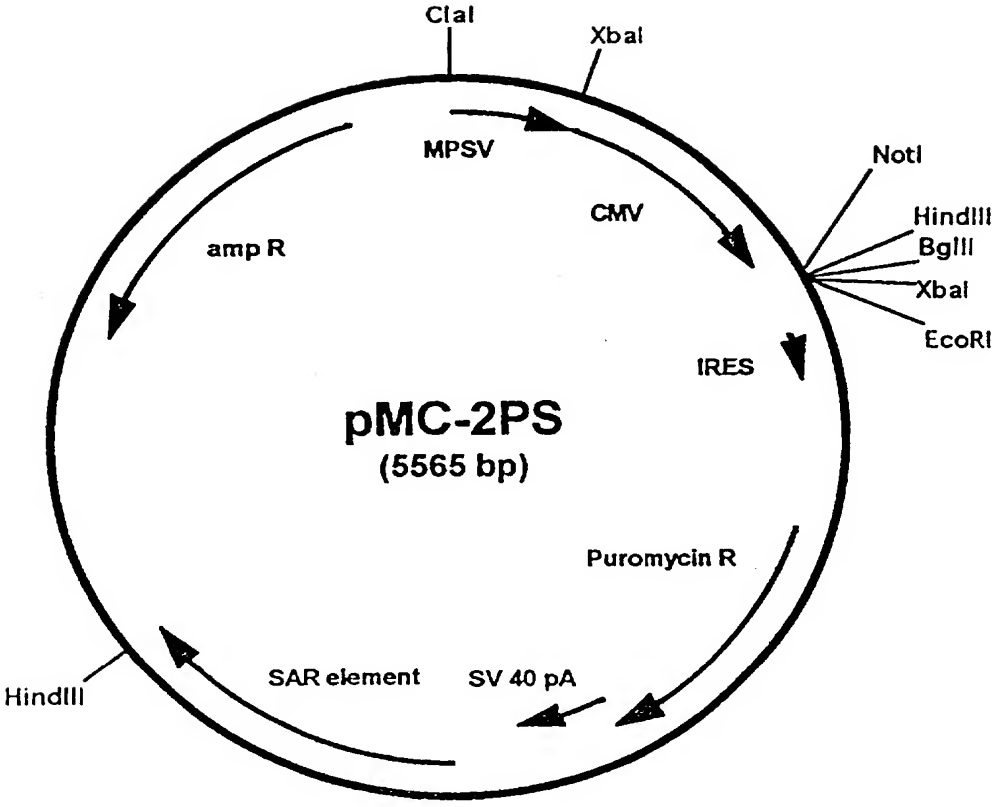
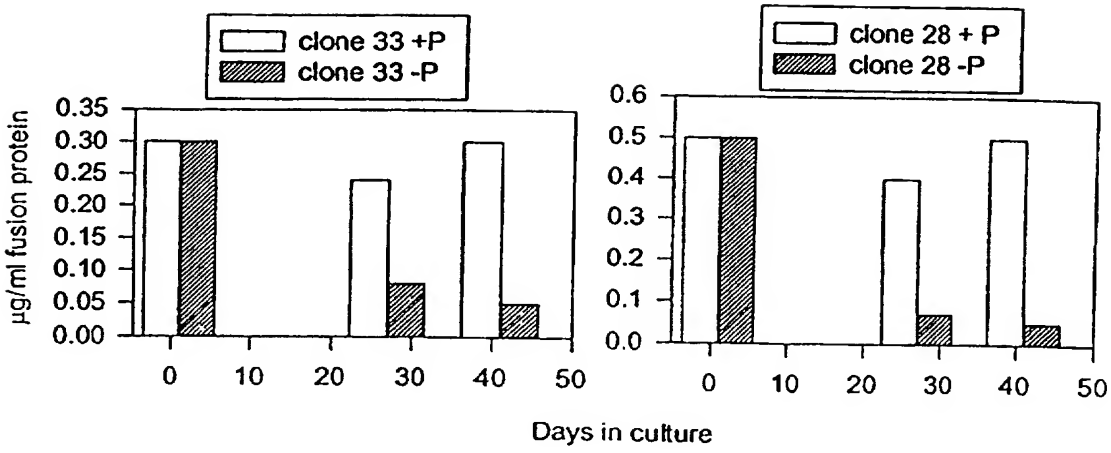

ClaI
XbaI
MPSV
NotI
CMV
HindIII
BglII
XbaI
EcoRI
amp R
IRES
pMC-2PS
(5565 bp)
Puromycin R
HindIII
SAR element
SV 40 pA

FIG. 1 G


clone 33 +P
clone 33 -P
clone 28 + P
clone 28 -P
µg/ml fusion protein
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Days in culture

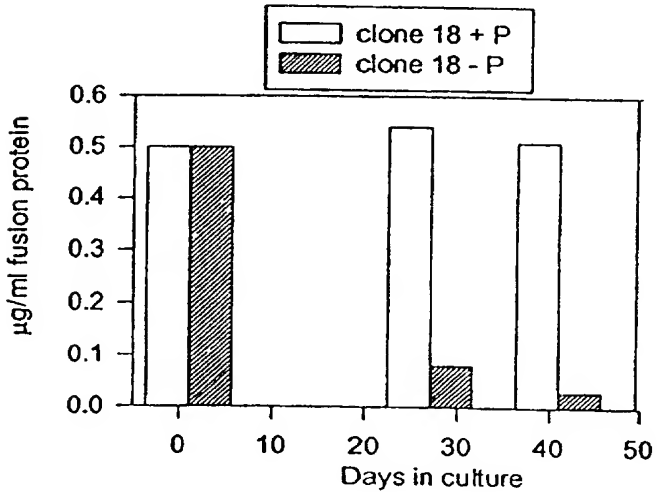

clone 18 + P
clone 18 - P
µg/ml fusion protein
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Days in culture

FIG. 2

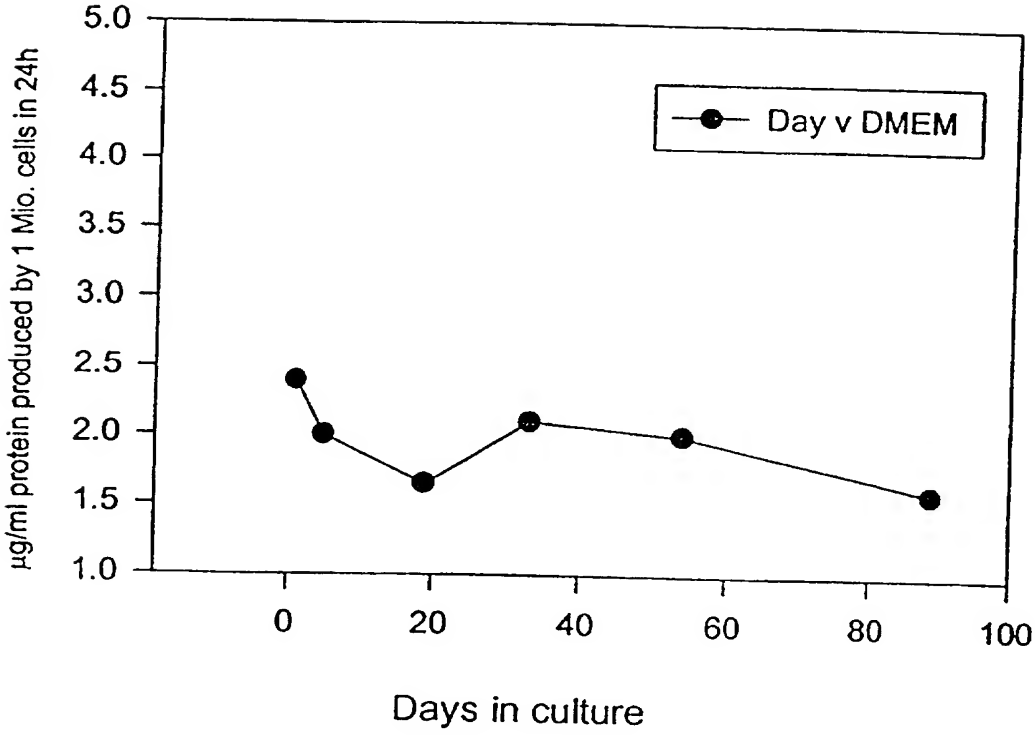

5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
µg/ml protein produced by 1 Mio. cells in 24h
Day v DMEM
0
20
40
60
80
100
Days in culture

FIG. 3

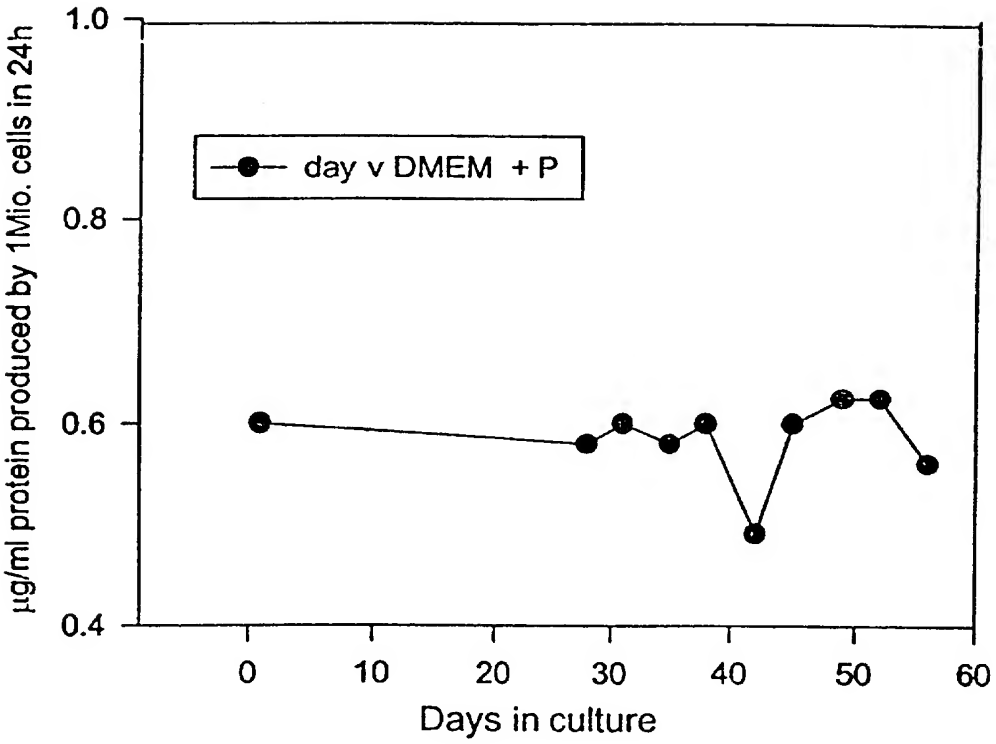

1.0
0.8
0.6
0.4
µg/ml protein produced by 1Mio. cells in 24h
day v DMEM + P
0
10
20
30
40
50
60
Days in culture

FIG. 4

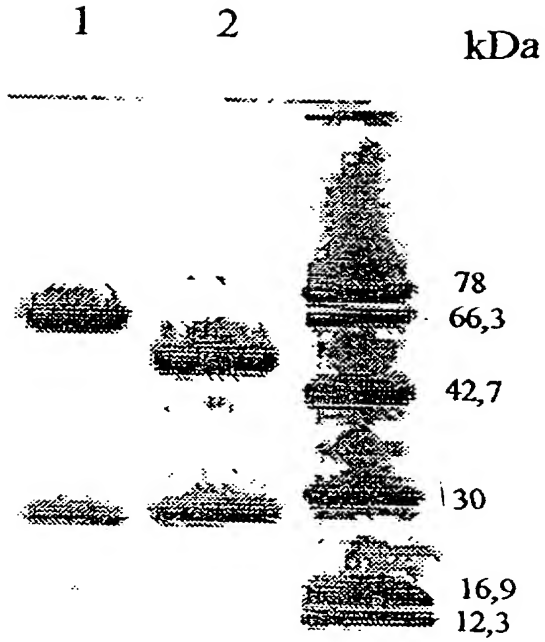


FIG. 5

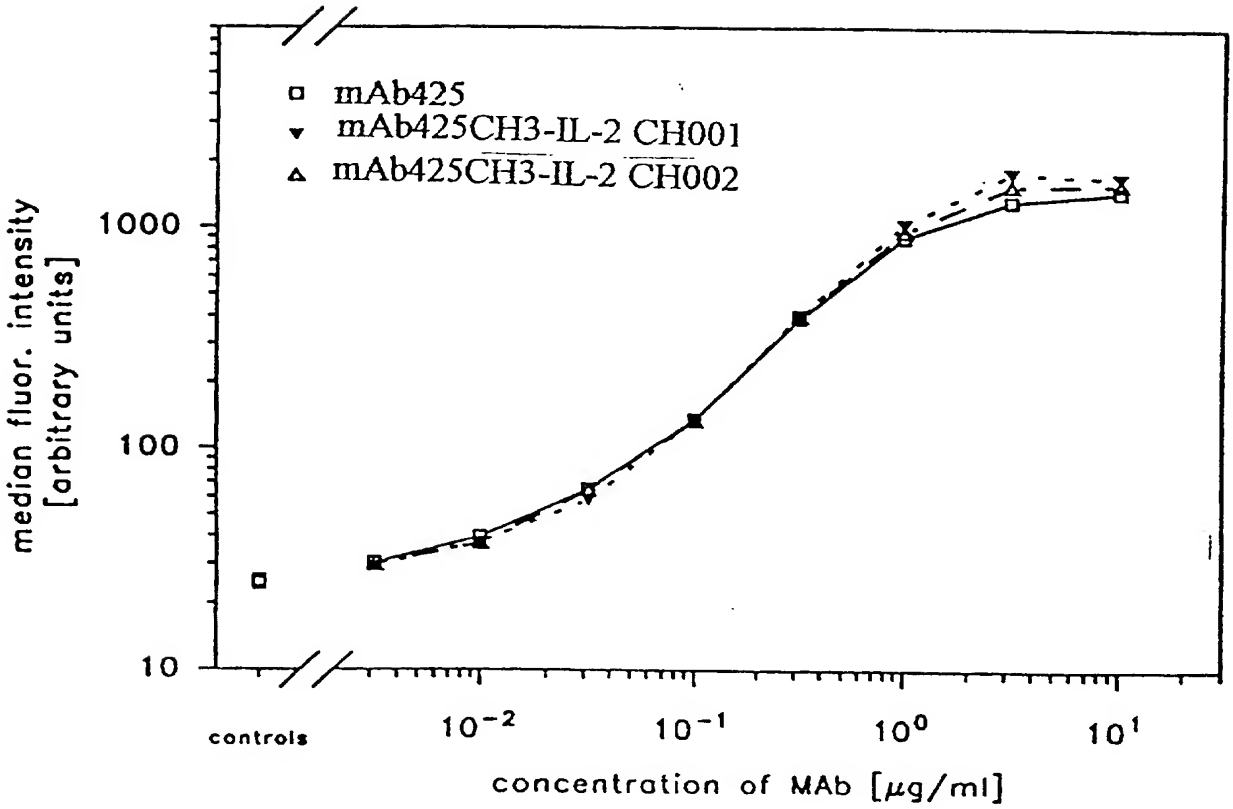

mAb425
mAb425CH3-IL-2 CH001
mAb425CH3-IL-2 CH002
median fluor. intensity [arbitrary units]
1000
100
10
controls
10^{-2}
10^{-1}
10^{0}
10^{1}
concentration of MAb [µg/ml]

FIG. 6

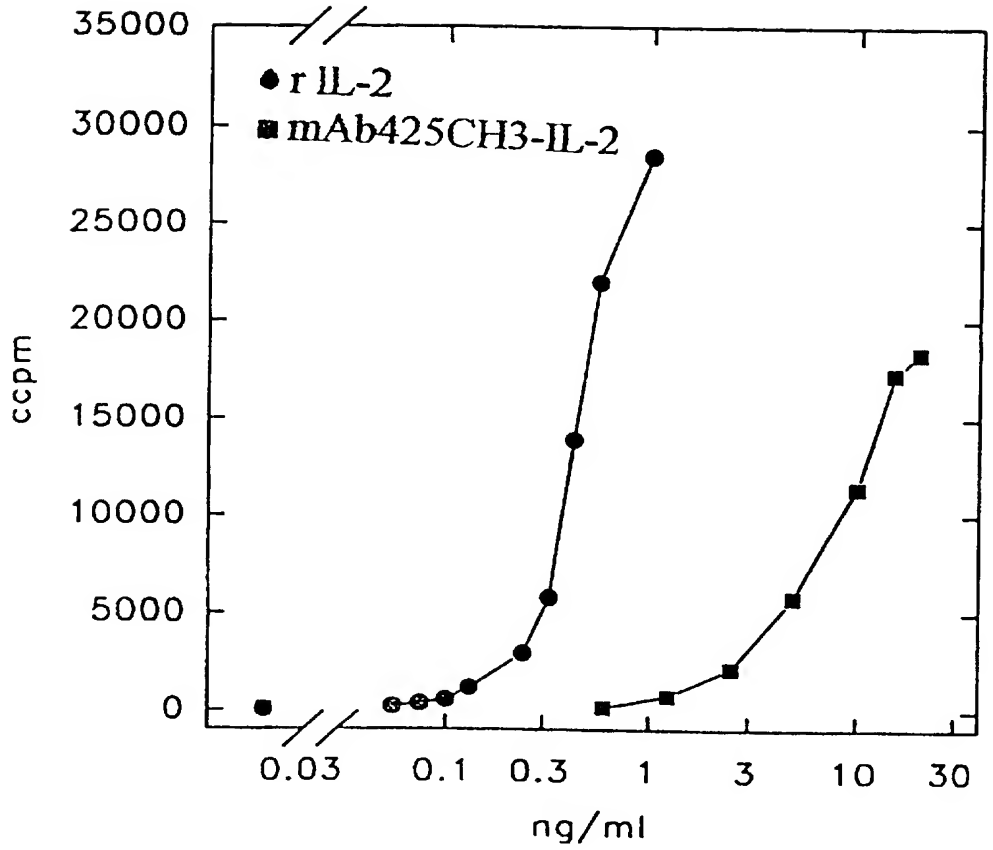

35000
30000
25000
20000
15000
10000
5000
0
ccpm
r IL-2
mAb425CH3-IL-2
0.03
0.1
0.3
1
3
10
30
ng/ml

FIG. 7

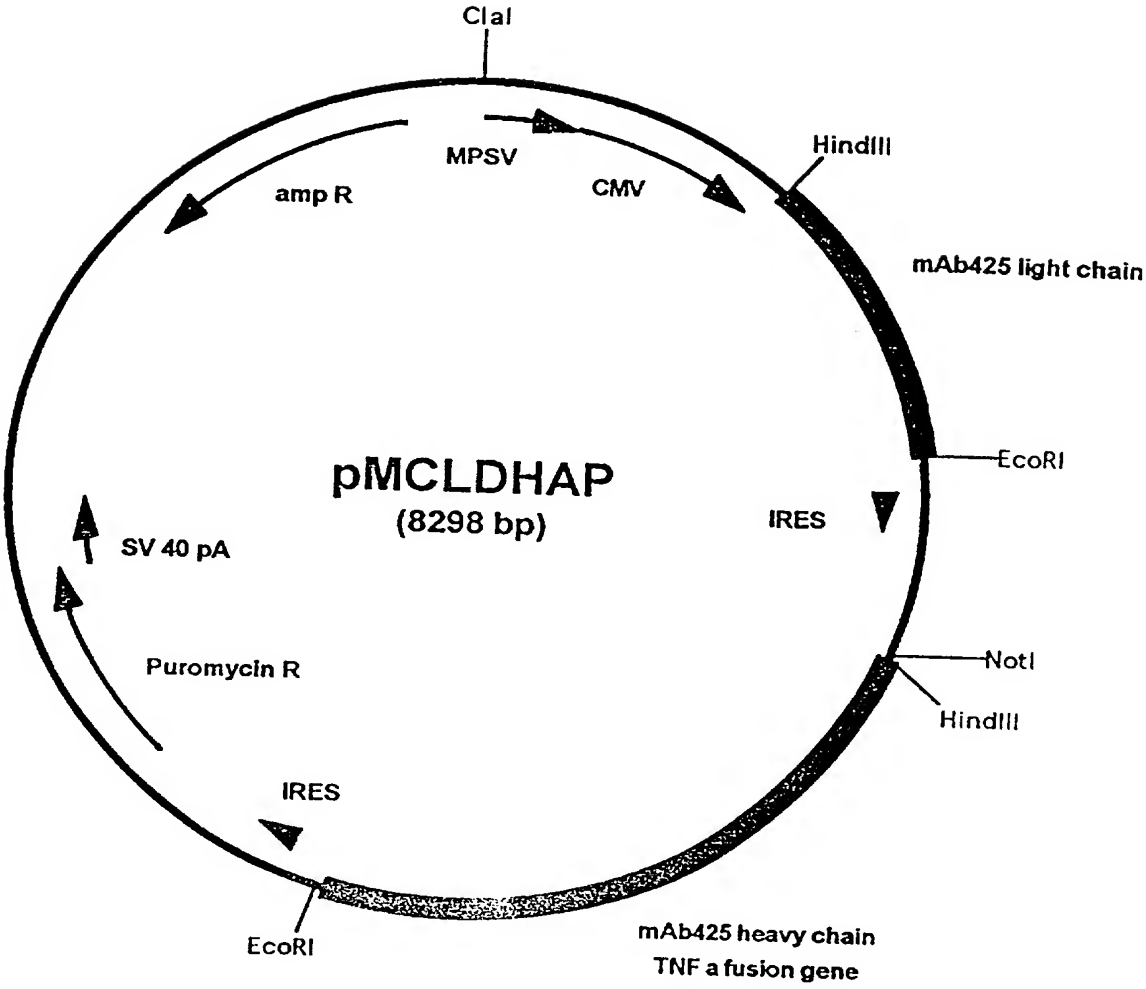

ClaI
MPSV
CMV
HindIII
amp R
mAb425 light chain
pMCLDHAP
(8298 bp)
EcoRI
IRES
SV 40 pA
Puromycin R
NotI
HindIII
IRES
EcoRI
mAb425 heavy chain
TNF a fusion gene

FIG. 8

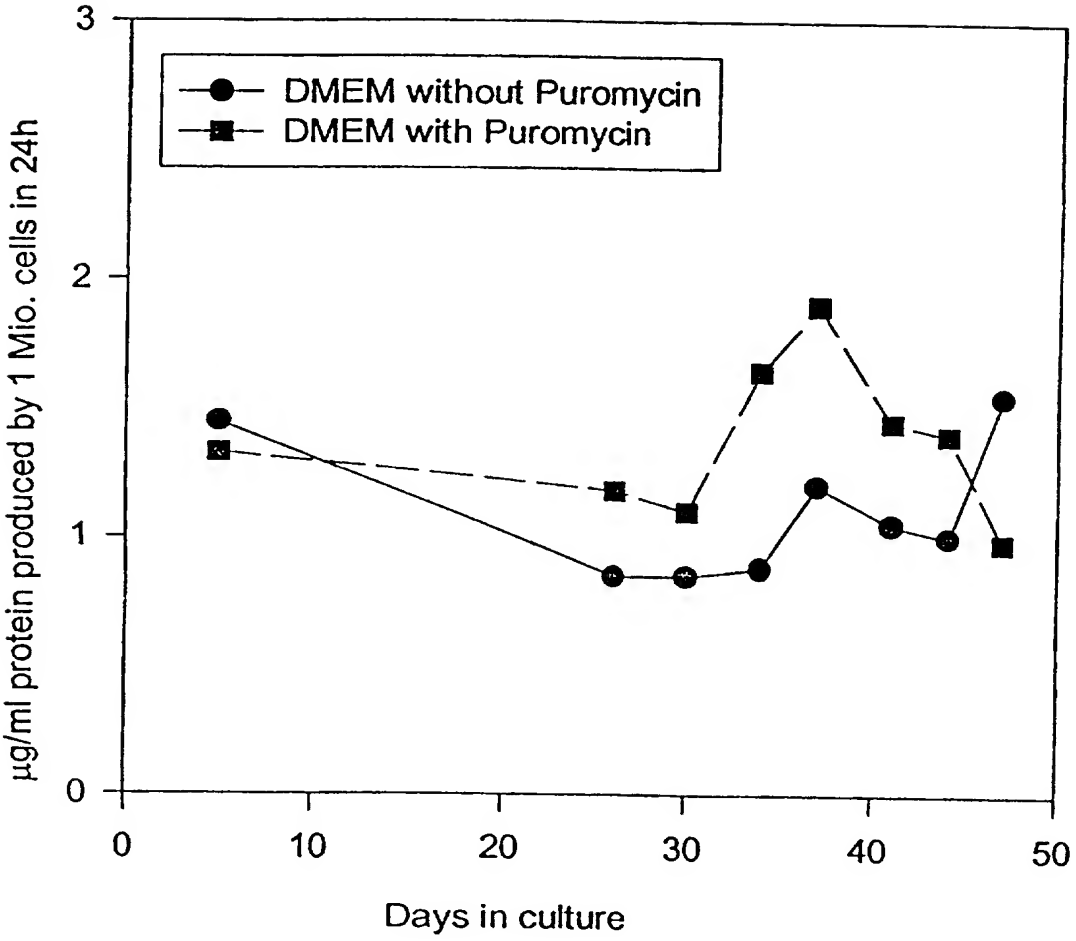

DMEM without Puromycin
DMEM with Puromycin
µg/ml protein produced by 1 Mio. cells in 24h
Days in culture
0
1
2
3
0
10
20
30
40
50

FIG. 9

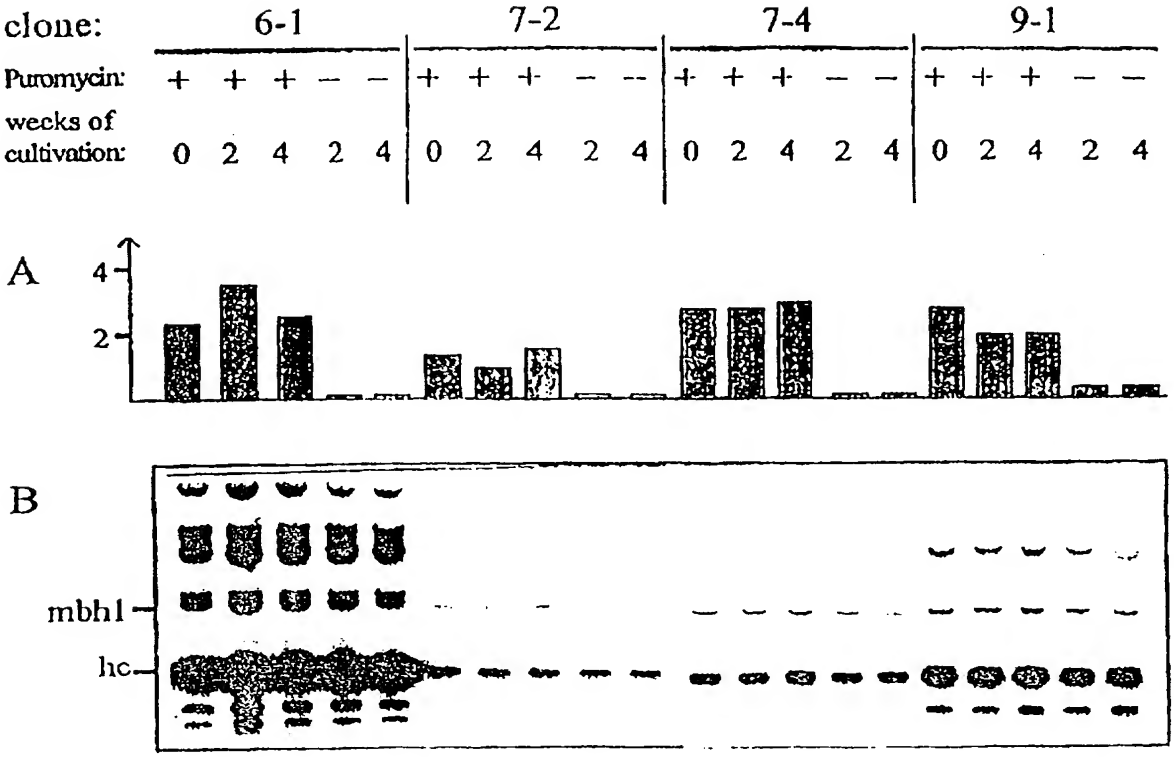

clone:
6-1
7-2
7-4
9-1
Puromycin:
+ + + – – + + + – – + + + – – + + + – –
weeks of cultivation:
0 2 4 2 4 0 2 4 2 4 0 2 4 2 4 0 2 4 2 4
A
4
2
B
mbh1
hc

FIG. 10

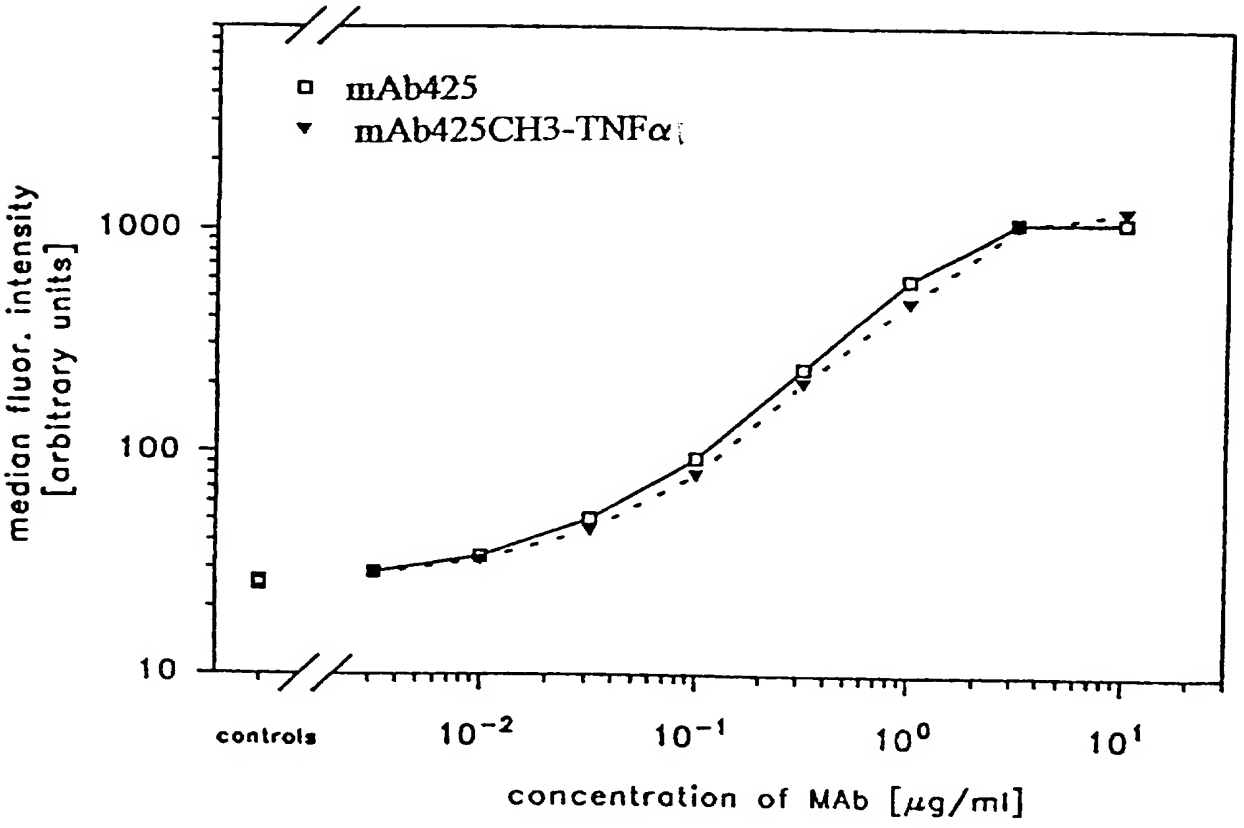
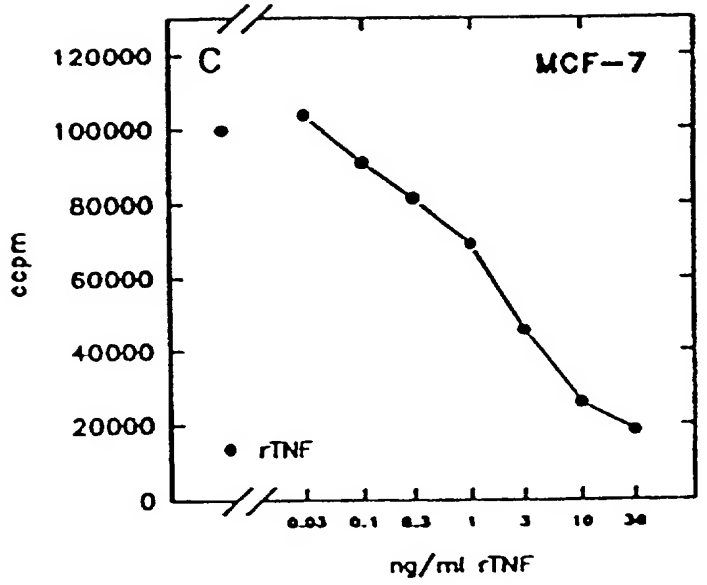

mAb425
mAb425CH3-TNFα
median fluor. intensity [arbitrary units]
1000
100
10
controls
10^{-2}
10^{-1}
10^{0}
10^{1}
concentration of MAb [μg/ml]

FIG. 11


C
MCF-7
120000
100000
80000
60000
40000
20000
0
ccpm
rTNF
0.03
0.1
0.3
1
3
10
30
ng/ml rTNF

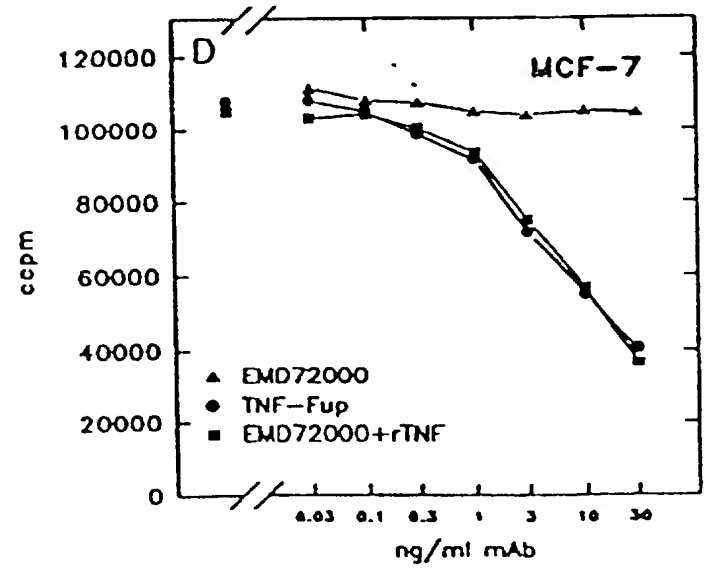

D
MCF-7
120000
100000
80000
60000
40000
20000
0
ccpm
EMD72000
TNF-Fup
EMD72000+rTNF
0.03
0.1
0.3
1
3
10
30
ng/ml mAb

FIG. 12

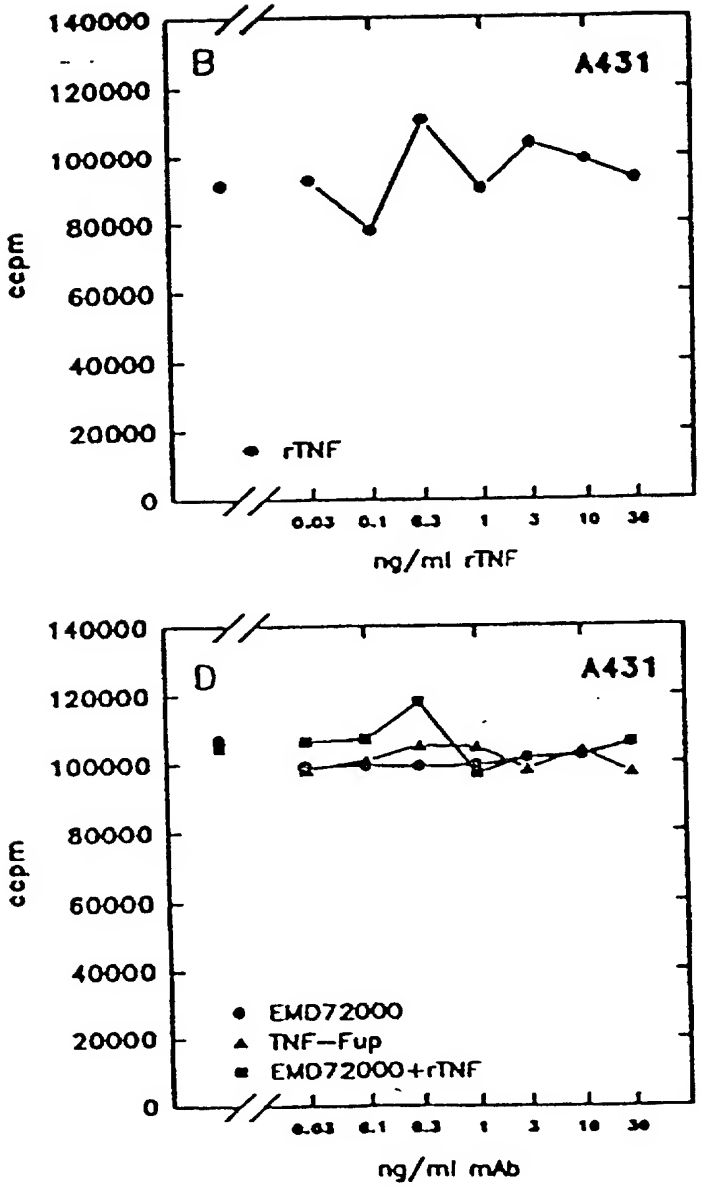

B
A431
140000
120000
100000
80000
60000
40000
20000
0
ccpm
rTNF
0.03
0.1
0.3
1
3
10
30
ng/ml rTNF
D
A431
EMD72000
TNF–Fup
EMD72000+rTNF
ng/ml mAb

FIG. 13

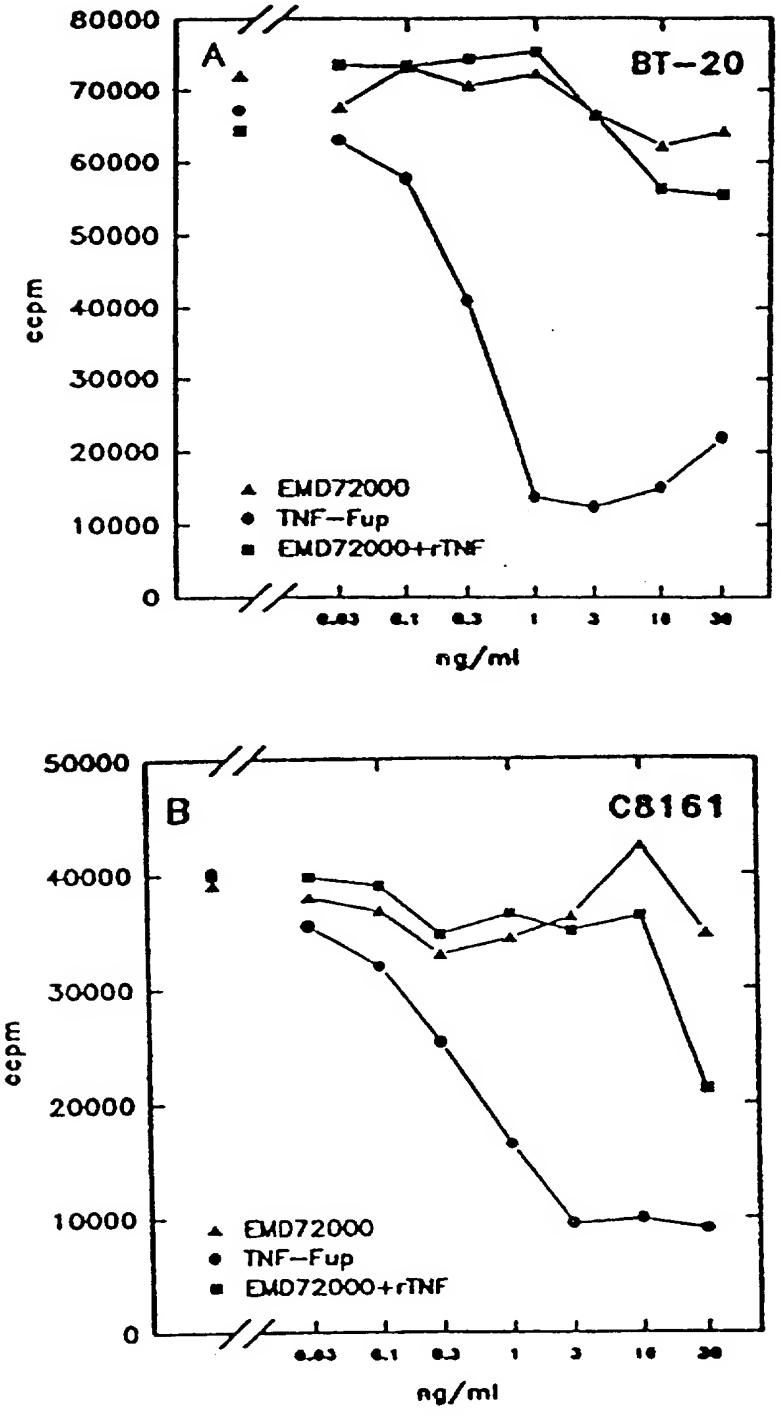

A
BT-20
80000
70000
60000
50000
40000
30000
20000
10000
0
ccpm
EMD72000
TNF-Fup
EMD72000+rTNF
0.03
0.1
0.3
1
3
10
30
ng/ml
B
C8161
50000
40000
30000
20000
10000
0
ccpm
EMD72000
TNF-Fup
EMD72000+rTNF
0.03
0.1
0.3
1
3
10
30
ng/ml

FIG. 14

Fig.: 15

TCGATAATGA AAGACCCCAC CTGTAGGTTT GGCAAGCTAG CTTAAGTAAC GCCATTTTGC 60

AAGGCATGGG AAAAATACAT AACTGAGAAT AGAGAAGTTC AGATCAAGGT CAGGAACAGA 120

GAAACAGGAG AATATGGGCC AAACAGGATA TCTGTGGTAA GCAGTTCCTG CCCCGCTCAG 180

GGCCAAGAAC AGTTGGAACA GGAGAATTGG GCCAAACAGG ATATCTGTGG TAAGCAGTTC 240

CTGCCCCGCT CAGGGCCAAG AACAGATGGT CCCCAGATGC GGTCCCGCCC TCAGCAGTTT 300

CTAGACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCATT 360

GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA 420

ATGGGTGGAG TATTTACGGT AAACTGCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC 480

AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA 540

CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC 600

CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG 660

ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC AAAATCAACG 720

GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT 780

ACGGTGGGAG GTCTATATAA GCAGAGCTCG TTTAGTGAAC CGTCAGATCG CCTGGAGACG 840

CCATCCACGC TGTTTTGACC TCCATAGAAG ACACCGGGAC CGATCCAGCC TCGAGGAACT 900

GGAAAACCAG AAAGTTAACT GGTAAGTTTA GTCTTTTTGT CTTTTATTTC AGGTCCCGGA 960

ATTAAGCTTC GCCACC ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA 1009
Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val

GCA ACA GCT AC AGGTAAGGGG CTCACAGTAG CAGGCTTGAG GTCTGGACAT 1060
Ala Thr Ala

ATATATGGGT GACAATGACA TCCACTTTGC CTTTCTCTCC ACAGGT GTC CAC TCC 1115
Val His Ser

GAC ATC CAG ATG ACC CAG AGC CCA AGC AGC CTG AGC GCC AGC GTG GGT 1163
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly

GAC AGA GTG ACC ATC ACC TGT AGT GCC AGC TCA AGT GTA ACT TAC ATG 1211
Asp Arg Val Thr Ile Thr Cys ***Ser Ala Ser Ser Ser Val Thr Tyr Met***

TAT TGG TAC CAG CAG AAG CCA GGT AAG GCT CCA AAG CTG CTG ATC TAC 1259
Tyr Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr

GAC ACA TCC AAC CTG GCT TCT GGT GTG CCA AGC AGA TTC AGC GGT AGC 1307
Asp Thr Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser

GGT AGC GGT ACC GAC TAC ACC TTC ACC ATC AGC AGC CTC CAG CCA GAG 1355
Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro Glu

GAC ATC GCC ACC TAC TAC TGC CAG CAG TGG AGT AGT CAC ATA TTC ACG 1403
Asp Ile Ala Thr Tyr Tyr Cys ***Gln Gln Trp Ser Ser His Ile Phe Thr***

TTC GGC CAA GGG ACC AAG GTG GAA ATC AAA CGTGAGTAGA ATTTAAACTT 1453
Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

TGCTTCCTCA GTTGGATCCA TCTGGGATAA GCATGCTGTT TTCTGTCTGT CCCTAACATG 1513

CCCTGTGATT ATGCGCAAAC AACACACCCA AGGGCAGAAC TTTGTTACTT AAACACCATC 1573

CTGTTTGCTT CTTTCCTCAG GA ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC 1625
Thr Val Ala Ala Pro Ser Val Phe Ile Phe

CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC 1673
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys

CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG AAG GTG 1721
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GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC ACA GAG CAG 1769
Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln

GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC CTG ACG CTG AGC 1817
Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser

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CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG TGT 1913
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CGGTGACGGT GGTCCAGGCT GCGTTGGCGG CCTACCTATG GCTAACGCCA TGGGACGCTA 2326

GTTGTGAACA AGGTGTGAAG AGCCTATTGA GCTACATAAG AATCCTCCGG CCCCTGAATG 2386

CGGCTAATCC CAACCTCGGA GCAGGTGGTC ACAAACCAGT GATTGGCCTG TCGTAACGCG 2446

CAAGTCCGTG GCGGAACCGA CTACTTTGGG TGTCCGTGTT TCCTTTTATT TTATTGTGGC 2506

TGCTTATGGT GACAATCACA GATTGTTATC ATAAAGCGAA TTGGATTGCG GCCGCGAATT 2566

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GTG GCT CCT GGG GCC CAC AGC CAG GTG CAA CTA GTG CAG TCC GGC GCC 2665
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GAA GTG AAG AAA CCC GGT GCT TCC GTG AAG GTG AGC TGT AAA GCT AGC 2713
Glu Val Lys Lys Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser

GGT TAT ACC TTC ACA TCC CAC TGG ATG CAT TGG GTT AGA CAG GCC CCA 2761
Gly Tyr Thr Phe Thr ***Ser His Trp Met His*** Trp Val Arg Gln Ala Pro

GGC CAA GGG CTC GAG TGG ATT GGC GAG TTC AAC CCT TCA AAT GGC CGG 2809
Gly Gln Gly Leu Glu Trp Ile Gly ***Glu Phe Asn Pro Ser Asn Gly Arg***

ACA AAT TAT AAC GAG AAG TTT AAG AGC AAG GCT ACC ATG ACC GTG GAC 2857
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ACC TCT ACA AAC ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG 2905
Thr Ser Thr Asn Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu

GAC ACT GCA GTC TAC TAC TGC GCC TCA CGG GAT TAC GAT TAC GAT GGC 2953
Asp Thr Ala Val Tyr Tyr Cys Ala Ser ***Arg Asp Tyr Asp Tyr Asp Gly***

AGA TAC TTC GAC TAT TGG GGA CAG GGT ACC CTT GTC ACC GTC AGT TCA 3001
Arg Tyr Phe Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser

GGT GAG TGG ATC CTC TGC GCC TGG GCC CAG CTC TGT CCC ACA CCG CGG 3049
Gly Glu Trp Ile Leu Cys Ala Trp Ala Gln Leu Cys Pro Thr Pro Arg

TCA CAT GGC ACC ACC TCT CTT GCA GCC TCC ACC AAG GGC CCA TCG GTC 3097
Ser His Gly Thr Thr Ser Leu Ala Ala Ser Thr Lys Gly Pro Ser Val

TTC CCC CTG GCA CCC TCC TCC AAG AGC ACC TCT GGG GGC ACA GCG GCC 3145
Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala

CTG GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG 3193
Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser

TGG AAC TCA GGC GCC CTG ACC AGC GGC GTG CAC ACC TTC CCG GCT GTC 3241
Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val

CTA CAG TCC TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG ACC GTG CCC 3289
Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro

TCC AGC AGC TTG GGC ACC CAG ACC TAC ATC TGC AAC GTG AAT CAC AAG 3337
Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys

CCC AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG CCC AAA TCT TGT GAC 3385
Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp

AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA 3433
Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly

CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC 3481
Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile

TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA 3529
Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu

GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT 3577
Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His

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Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg

GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG 3673
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Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu

AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC 3769
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ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG 3817
Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu

ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG 3865
Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp

GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG 3913
Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val

CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC 3961
Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp

AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT 4009
Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His

GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG 4057
Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro

GGT AAA ATG GTC AGA TCA TCT TCG CGA ACC CCG AGT GAC AAG CCT GTA 4105
Gly Lys **Met** Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val

GCC CAT GTT GTA GCA AAC CCT CAA GCT GAG GGG CAA CTG CAG TGG CTG 4153
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Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp

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GTC CTC TTC AAG GGC CAA GGC TGC CCG TCG ACC CAT GTG CTC CTC ACC 4297
Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr

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CTC TCT GCC ATC AAG AGC CCC TGC CAG AGG GAG ACC CCA GAG GGG GCT 4393
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Ala Trp Phe Leu Ala Thr Val Gly Val Ser Pro Asp His Gln Gly Lys

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GCC GGG GTG CCC GCC TTC CTG GAG ACC TCC GCG CCC CGC AAC CTC CCC 5786
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ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT ATTATCATGA CATTAACCTA 8219

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FIG. 16

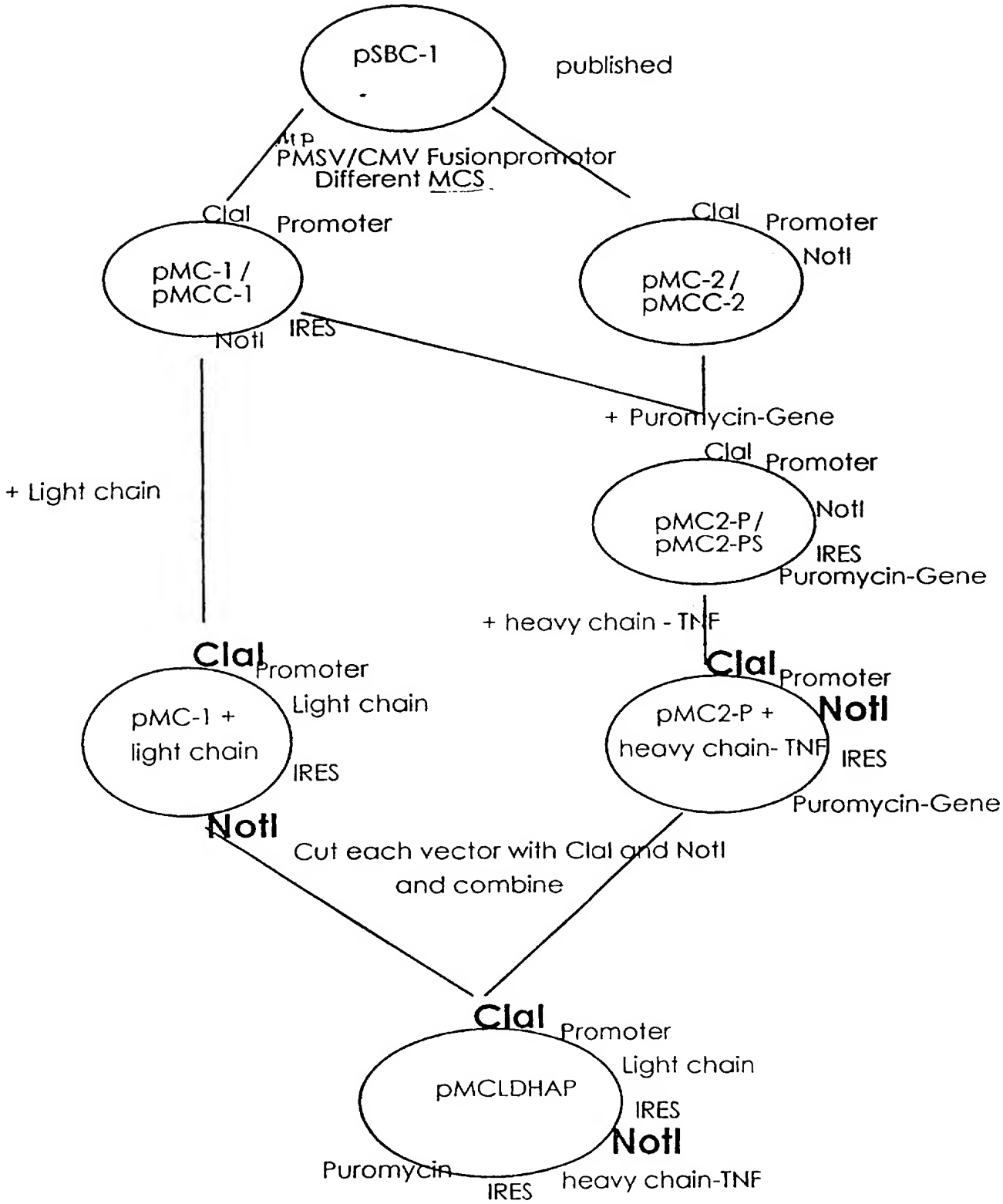
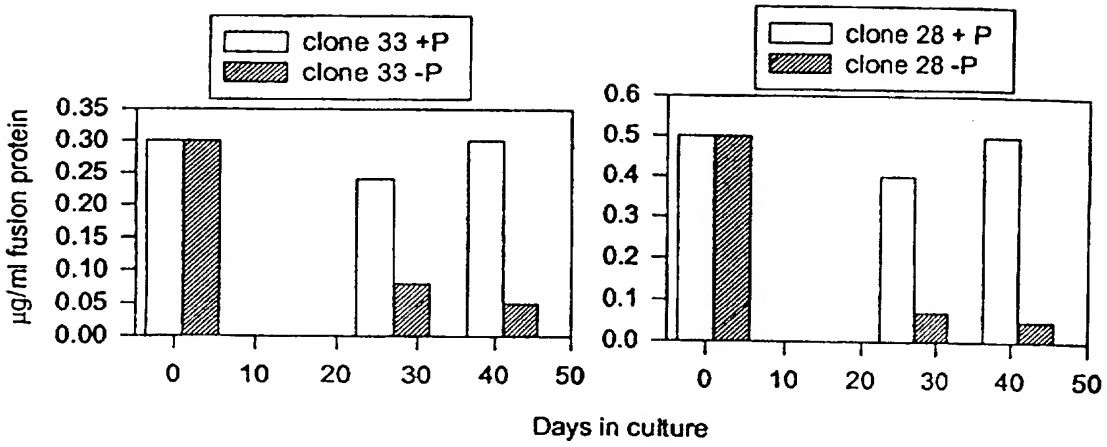

pSBC-1
published
PMSV/CMV Fusionpromotor
Different MCS
ClaI
Promoter
pMC-1 /
pMCC-1
NotI
IRES
ClaI
Promoter
NotI
pMC-2 /
pMCC-2
+ Puromycin-Gene
+ Light chain
ClaI
Promoter
pMC2-P /
pMC2-PS
NotI
IRES
Puromycin-Gene
+ heavy chain - TNF
ClaI
Promoter
Light chain
pMC-1 +
light chain
IRES
NotI
ClaI
Promoter
NotI
pMC2-P +
heavy chain- TNF
IRES
Puromycin-Gene
Cut each vector with ClaI and NotI
and combine
ClaI
Promoter
Light chain
pMCLDHAP
IRES
NotI
Puromycin
IRES
heavy chain-TNF

FIG. 17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clone 33 -P
clone 28 + P
clone 28 -P
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0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Days in culture

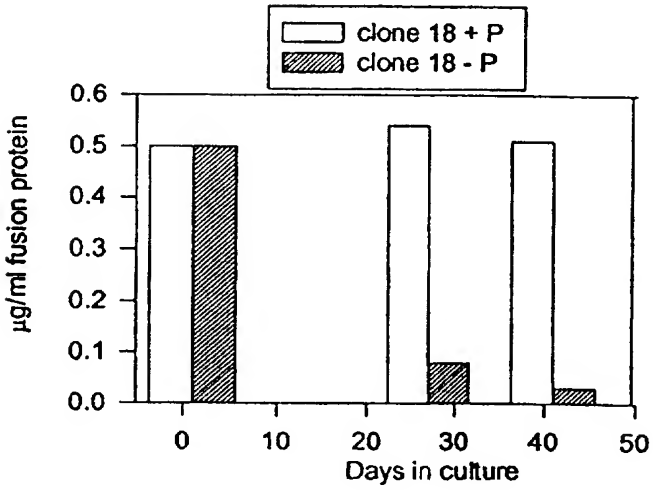
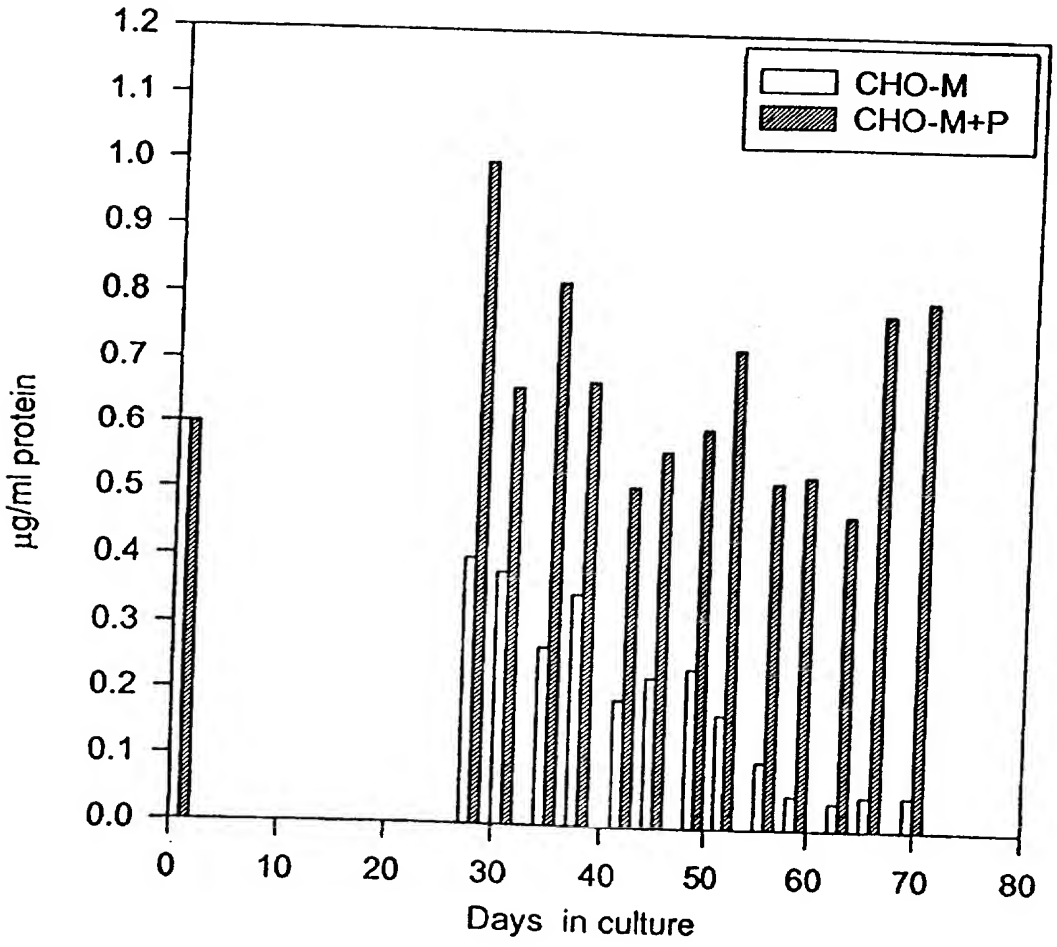

clone 18 + P
clone 18 - P
µg/ml fusion protein
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
Days in culture

FIG. 18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CHO-M+P
µg/ml protein
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
Days in culture

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/04765

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/13 C07K16/28 C12N15/43 C12N15/28
C07K14/525 C12N15/26 C07K14/55 C12N15/19 C07K14/52
C12N5/10 C12N15/62 C07K19/00 G01N33/53 G01N33/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 24870 A (BIOTRANSPLANT INC ;GEN HOSPITAL CORP (US); LE GUERN CHRISTIAN A (U) 10 November 1994	1,2,13, 15
Y	see page 11, paragraph 3 - page 12, paragraph 3 see page 18, paragraph 3 - page 19, paragraph 2; figures 1J-L ---	3-12,14, 16
Y	EP 0 659 439 A (MERCK PATENT GMBH) 28 June 1995 cited in the application see abstract see page 3, line 20 - page 4, line 7 see page 6, line 20 - page 9; table 1 see page 11 - page 12; claims see page 13; figure 1 --- -/--	3-12,14, 16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1997	23/01/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Macchia, G

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/04765

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NOEL D. ET AL.: "Analysis of the individual contributions of immunoglobulin heavy and light chains to the binding of antigen using cell transfection and plasmon resonance analysis" JOURNAL OF IMMUNOLOGICAL METHODS, vol. 193, no. 2, 21 June 1996, page 177-187 XP004020811 see page 180; figure 1 see page 182; figure 2 --- -/--	3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1997	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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